치의학박사학위논문

Development of a totalplex amplification and bead array-based genotyping method for killer cell immunoglobulin-like receptor

다중유전자 증폭 및 비드 어레이 기술을 이용한 자연살해세포 면역글로불린 유사 수용체 유전형 동정 방법 개발

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서울대학교 대학원
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ABSTRACT

Development of a totalplex amplification and bead array-based genotyping method for killer cell immunoglobulin-like receptor

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Natural killer cells have Killer cell Immunoglobulin-like Receptors (KIR) that interact with human class I leukocyte antigens. The KIR genes belong to a multigene family that contains 16 members which display a high level of sequence similarity. The composition of KIR genes in each individual is highly polymorphic so that there can be 186 different genotypes. Recently, it has been reported that the interactions between KIR and human leukocyte antigens affect infection resistance, autoimmune disease, cancer, reproduction and the outcome of hematopoietic stem cell transplantation, increasing the importance of KIR genotyping. Among the various KIR genotyping methods currently used, sequence-specific primer directed polymerase chain reaction

(SSP-PCR) technology has been widely used because of its simplicity. But it is a time- and labor-consuming method and needs genomic DNA at the μg level. In addition, SSP-PCR method has the disadvantage of being partially or totally based on the amplification of long DNA fragments which requires high quality DNA. Therefore, it is necessary to develop more sensitive, accurate and less sample consuming methods for KIR genotyping. In this study, I developed a novel technique for KIR genotyping using totalplex amplification and a Luminex bead array. In stage of KIR gene amplification, totalplex amplification was performed to amplify 16 KIR genes using specific bulge specific primers in two separate reactions. Then amplified products were mixed and labeling was performed using gene specific primer extension primers. Amplified KIR gene signals were detected using Luminex bead array analysis and then the results were translated to individual KIR genotypes by median fluorescence intensity. For validation of totalplex/bead array-based KIR genotyping, a NK/KIR reference panel I consisting of 48 cell types provided by the 13th the International Histocompatibility Working Group was used. The KIR genotyping results of the totalplex/bead array-based method coincided with the information provided with the NK/KIR reference panel I. When KIR genotyping was performed using the DNA samples from 8 human cell lines, 8 buccal samples and 17 peripheral blood samples, the results from the newly developed method had a 100% correlation with those obtained by SSP-PCR. The reproducibility of the newly developed method was verified by showing that 6 repetitive genotypings of the same DNA samples produced the same KIR genotype results. The sensitivity of the method was confirmed by

demonstrating that accurate KIR genotyping results were obtained when DNA samples of 5 ng or more were used. These results indicate that the newly developed totalplex/bead array-based KIR genotyping method is a sensitive and reproducible method that has the advantage of saving on reagent cost, time, labor and DNA amount necessary for KIR genotyping.

Keywords: Killer cell immunoglobulin-like receptors, genotyping, specific bulge specific primer, totalplex amplification, bead array, gene specific primer extension amplification

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INTRODUCTION

Human natural killer (NK) cells are bone marrow-derived lymphocytes that are crucial components of the innate immune system (Trinchieri, 1989). NK cell functions are controlled by diverse families of antigen receptors and host cells expressing HLA class 1 antigens (Parham, 2005). The genes encoding killer cell immunoglobulin-like receptors (KIRs), a family of genes clustered in 19q3.4, are among NK cell receptors (Middleton and Gonzelez, 2009).

The KIR genes have either two or three extracellular immunoglobulin domains, called 2D or 3D and either a long (L) or short (S) intracellular domain (Wagtmann *et al.*, 1995; Vilches and Parham, 2002). In general, the KIR having long cytoplasmic tails transduce inhibitory signals whereas the KIR having short cytoplasmic tails transduce activating signals. KIR haplotypes can be split into two basic groups termed haplotypes A and B based on gene content (Hsu *et al.*, 2002b; Kulkarni *et al.*, 2008). The most common haplotype, group A, contains five inhibitory genes, KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2, and KIR3DL3 and two activating KIR genes, KIR2DS4 and KIR2DL4. Previous studies have reported that KIR2DL4 may have both inhibitory and activating capacity (Uhrberg *et al.*, 1997; Wilson *et al.*, 2000). B haplotypes are characterized by variable gene numbers (Figure 1). The alloreactions of human NK-cells are defined mainly by the activity of inhibitory receptors with specificity for human leukocyte antigen (HLA) class I. There are interaction with KIR and HLA, HLA-A and

KIR3DL2, HLA-B and KIR3DL1, HLA-C and KIR2DL1, and KIR2DL2 and KIR2DL3 (Figure 2).

Inhibitory receptors with specificity for HLA class I appear to be important mediators of self-tolerance for NK cells (Long, 1999). The interaction of KIR2DL1 with HLA-C2 results in strong inhibition that is difficult to overcome by simultaneous activating signals (Winter *et al.*, 1998). Thus there is no killing of the target. On the other hand, KIR2DL3–HLA-C1 interaction is weaker and can be overridden by signals through activating receptors upon appropriate ligand binding, resulting in lysis of the target (Moesta *et al.*, 2008). The activating receptors KIR2DS2, 2DS1 and 3DS1 are thought to exhibit ligand specificity similar to the corresponding inhibitory counterparts, although their interactions are much weaker (Winter and Long, 1997). Allelic variation also plays a role in determining the strength of the interaction (Yawata *et al.*, 2006; Ahlenstiel *et al.*, 2008; Kulkarni *et al*., 2008).

The number and type of KIR genes are variable among individuals, and many studies increasingly report a link between KIR genotypes and certain human diseases (Ashouri *et al.*, 2009). KIR polymorphism modulates the intensity and quality of innate and adaptive immune response, thus affecting human health. For example, various KIR and HLA class I molecules have been attributed to infection resistance, autoimmune disease, cancer, reproduction and the facility of hematopoietic stem cell transplantation (Hsu *et al.*, 2002a; Parham, 2005; Khakoo and Carrington, 2006; Kulkarni *et al*., 2008; Velardi, 2008). When chronic myelogenous leukemia patients were transplanted with an HLA-C-mismatched, and KIR epitope-matched

hematopoietic stem cells from unrelated donors, they had longer total and disease-free survival than patients transplanted with cells mismatched in both HLA-C and KIR epitopes (Schaffer *et al.*, 2003). Therefore, KIR genotyping is important to select appropriate transplant donor.

Preeclampsia is a serious complication of pregnancy affecting the mortality and morbidity of both mothers and infants. It is characterized by hypertension and proteinuria that develop after 20 weeks of gestation in previously normotensive women (Redman and Sargent, 2005; Sibai *et al.*, 2005). Although the primary mechanism of preeclampsia is still unknown, it often shows impaired placental function, abnormal trophoblast invasion, deficient physiologic maternal spiral artery modification, increased apoptosis of trophoblastic cells and placental ischemia (Meekins *et al.*, 1994; Aldrich *et al.*, 2000). A genetic contribution from the male partner is known to be important in development of preeclampsia (Vatten and Skjaerven, 2003; Maconochie *et al.*, 2007). Previous reports have shown that pregnant woman who are homozygous for KIR haplotype A and carry a fetus that expresses HLA-C2 are at high risk of preeclampsia (Hiby *et al.*, 2004). Of the possible combinations of HLA-C and KIR2DL molecules, HLA-C2 with KIR2DL1 is the combination expected to provide the strongest inhibition (Parham, 2004). In this combination, HLA-C2 at the cell surface of extravillous trophoblasts can engage KIR2DL1 expressed by uterine NK cells and generate inhibitory signals. This indicates a model in which preeclampsia is caused by overly inhibited NK cells. Therefore, the genotyping of KIR in the mother and HLA in the father has value as a possible predictor for preeclampsia. The importance of KIR genotyping has thus increased, and various KIR

genotyping technologies have been developed using sequence-specific oligonucleotide probes (SSOP) (Crum *et al.*, 2000), reverse sequence-specific oligonucleotides (rSSO) (Nong *et al.*, 2007), MALDI-TOF mass spectrometry (Houtchens *et al.*, 2007), magnetic bead-bound oligonucleotide probes (Roberts *et al.*, 2007), and pyrosequencing (Yun *et al.*, 2007). Sequence-specific primer directed polymerase chain reaction (SSP-PCR) technology has also been applied to identify the presence and absence of the 16 KIR genes (Sun *et al.*, 2004; Vilches *et al.*, 2007) and still widely used because of its simplicity. However, these are time-and labor-consuming and need genomic DNA at the μg level. In addition, SSP-PCR methods have the disadvantage of being partially or totally based on the amplification of long DNA fragments, which requires high quality DNA.

Therefore, it is necessary to develop more sensitive and accurate methods for KIR genotyping. In this study, I developed a novel technique for KIR genotyping totalplex amplification with specific bulge specific (SBS) primers followed by Gene Specific Primer Extension (GSPE) and Luminex bead array analysis.

LITERATURE REVIEW

NK cells

NK cells play a key role in the immune response to viral infections and malignancies (Orr and Lanier, 2010). They can lyse target cells by recognizing major histocompatibility complex (MHC)/HLA molecules on the target cells (Trinchieri, 1989). NK cells have highly specific target cell recognition mechanisms and have been traditionally classified as innate immune cells (Bancroft, 1993). However, recent reports suggest that similar to B and T cells, NK cells may also display immunological memory (O'Leary *et al.*, 2006; Cooper *et al*., 2009; Sun *et al.*, 2009). Although NK cells mainly mediate cell killing, they can also influence other immune cells by secreting cytokines (Johansson *et al.*, 2005). Genetic evidence also indicates associations between the genes and alleles of KIRs and the development of autoimmune diseases, and the outcome of pregnancy and infections, suggesting a role for NK cells in many different diseases and fundamental biological processes (Khakoo and Carrington, 2006).

Dominant control of NK cell activation is mediated by inhibitory receptors on the membrane of NK cells that bind to HLA class I molecules on surrounding cells. Because normal cells express abundant MHC class I molecules that bind to inhibitory receptors and are recognized as ‘self’, these cells can resist NK cell attacks. However, when cells lose expression of one or more HLA class I alleles, they often become NK cell targets. This process is

known as missing-self recognition and has been observed in allogeneic stem cell transplantation, in which it can contribute to a potent graft-versus-leukemia effect and improve patient survival (Ljunggren *et al.*, 1990; Ruggeri *et al.*, 2002; Karre *et al.*, 2005).

KIR

KIRs help human NK cells counteract infections by pathogens that evade the immune system by inducing down-regulation of HLA class I molecules in infected cells (Huard and Fruh, 2000; Lopez-Botet *et al.*, 2001). The KIR family recognizes different HLA class I ligands and exhibits either inhibitory or activating functions (Winter and Long, 1997). KIRs are structurally and functionally diverse receptors encoded by a family of polymorphic genes (Vilches and Parham, 2002). Until novo, 14 KIR genes and 2 pseudogenes have been described. The KIR gene is conserved throughout primates, suggesting that it is the ancestral gene from which all other KIRs are derived (Sambrook *et al.*, 2006). The KIR gene cluster is present on chromosome 19q13.4 within the leukocyte receptor complex and is flanked by KIR3DL3 at the centromeric end and KIR3DL2 at the telomeric end (Carrington and Martin, 2006). Two basic haplotypes have been defined on the basis of gene content, and are termed haplotypes A and B. Haplotype A is uniform in terms of gene content and is composed of five inhibitory genes, KIR2DL1, 2DL3, 3DL1, 3DL2 and 3DL3; and one activating gene KIR2DS4; and KIR2DL4, which may have both inhibitory and activating capacity. KIR2DL4 has a charged amino acid in the transmembrane domain that interacts with the

activating motif (ITAM) (Kikuchi-Maki *et al.*, 2005). Interestingly, many A haplotypes possess null variants of both KIR2DS4 and KIR2DL4 that are not expressed on the cell surface (Witt *et al.*, 2000; Hsu *et al*., 2002b). Thus, these haplotypes technically possess no functional activating KIR. The B haplotypes contain variable numbers of activating and inhibitory receptors and are the primary contributors to the extraordinary differences in gene profiles observed in distinct ethnic populations across the world (Single *et al.*, 2007).

HLA

Human leukocyte antigen, HLA, is the name of MHC in humans. HLA genes map to chromosome 6 and encode cell-surface antigen presenting proteins (Shiina *et al.*, 2004). The three main HLA class I genes are known as HLA-A, HLA-B, and HLA-C. The proteins produced from these genes are present on the surface of almost all cells. On the cell surface, these proteins are bound to protein fragments (peptides) that have been exported from within the cell. HLA class I proteins display these peptides to the immune system (Marsh, 2004). The HLA-C gene encodes ligands for the KIR2DL receptor, whereby a functional dimorphism determines KIR specificity. HLA-C1 gene alleles encode Ser77/Asp80 of the HLA-Cw α1 domain, and HLA-C1 binds to the inhibitory receptors KIR2DL2 and KIR2DL3 and probably also to the activating KIR2DS2 (Rajagopalan and Long, 2005; Anfossi *et al.*, 2006). The HLA-C2 gene alleles encode Asp77/Lys80 and bind to KIR2DL1 and possibly to KIR2DS1 (Rajagopalan and Long, 2005; Anfossi *et al*., 2006).

Compound genotypes of KIR and HLA-C contribute to susceptibility or resistance to a variety of infectious diseases and cancer (Liao *et al.*, 1991; Furukawa *et al.*, 1999).

SSP-PCR

SSP-PCR is the first method to estimate the frequencies of haplotypic combinations of KIR genes (Uhrberg *et al.*, 1997). SSP-PCR technology has also been applied to identify the presence and absence of the 16 KIR genes (Sun *et al*., 2004; Vilches *et a*l., 2007) and is still widely used because of its simplicity. Many KIR sequences have also been described since KIR genotyping by SSP-PCR was devised (Shilling *et al.*, 1998; Vilches *et al.*, 2000; Rajalingam *et al.*, 2001; Whang *et al.*, 2005; Roberts *et al*., 2007; Vilches *et al*., 2007; Ashouri *et al*., 2009; Kulkarni *et al.*, 2009; Bao *et al.*, 2010). KIR genotyping is performed using 16 SSP-PCR reactions per individual, each reaction being specific for a single KIR gene or pseudogene. Most reactions contain one forward and one reverse KIR specific primer. Therefore, SSP-PCR can only be used when the combination of polymorphisms tested are unique to the gene of interest. SSP-PCR specificity is hard to achieve across large regions of DNA (Vilches *et al*., 2007). As the KIR genes can be up to 16 kb in length, it is not always possible to implement SSP-PCR for their genotyping.

Multiplex PCR

Considerable time and effort can be saved by simultaneously amplifying multiple sequences in a single reaction, a process referred to as multiplex PCR. Recently, multiple PCR amplification has been more widely used than single PCR amplification. Multiplex PCR requires primers that amplify unique regions of DNA, either in a pair or in combinations of multiple primers, under a single set of reaction conditions. In addition, the method must be available for the analysis of each amplification product from the mixture of all the amplification products. Multiplex PCR is becoming a rapid and convenient screening assay in both clinical and research laboratories. However, the development of efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize the reaction conditions. For successful multiplex PCR assay, relative concentrations of the primers, concentration of the PCR buffer, a balance in the magnesium chloride and deoxynucleotide concentration, cycling temperature, amount of template DNA, and Taq DNA polymerase all have an important role. An optimal combination of annealing temperature and buffer concentration is essential in multiplex PCR to obtain highly specific amplification products. Magnesium chloride concentration needs to be proportional to the amount of dNTPs while adjusting the primer concentration for each target sequence is also essential. The list of various factors that can influence the reaction is by no means complete (Edwards and Gibbs, 1994).

Preeclampsia

Preeclampsia is a pregnancy specific heterogenic multisystem disorder characterized by hypertension (>140/90 mm Hg) and proteinuria (>300 mg/24 h) that develops after 20 weeks of gestation in a normal woman (Jebbink *et al.*, 2012). Preeclampsia is responsible for about 18% of maternal deaths and up to 40% of fetal mortalities (Anderson *et al.*, 2011). At this time, preeclampsia still lacks a safe and effective therapy. The only true therapeutic option is delivery, but at an early gestational age this forms a risk for the newborn. Premature delivery is a major risk factor for perinatal death and morbidity (Larroque *et al.*, 2004; Moster *et al.*, 2008). The disease evolves in two stages. The first stage is characterized by an altered formation of the placenta caused by maternal constitutional factors (genetic, behavioral or environmental) released from the placenta that are usually considered a toxin which reduces nutrient availability. Maternal constitutional factors contribute to a reduction in placental perfusion and then reduced perfusion, posited as secondary to failed remodeling of the maternal vessels leading to the maternal abnormalities of preeclampsia, stage 2 (Roberts and Hubel, 2009). During placentation, a defective invasion of the extravillous trophoblast cells into the muscle layers of the spiral arteries has been shown (Parham, 2004). Although the primary mechanism of preeclampsia is still unknown, it often shows impaired placental function, abnormal trophoblast invasion, deficient physiologic maternal spiral artery modification, increased apoptosis of trophoblastic cells and placental ischemia (Meekins *et al*., 1994; Aldrich *et al*., 2000).

MATERIALS AND METHODS

I. DNA samples

Genomic DNAs from 8 human cell lines, 8 human buccal samples and 17 peripheral blood samples (approved by the Institutional Review Board of Seoul National University Hospital, H-0904-011-277) were extracted using either a genomic DNA extraction kit (Komabiotech, Seoul, Korea) or a buccal cell genomic DNA isolation kit (YeBT, Seoul, Korea). 48 DNA samples of NK/KIR reference panel I were obtained from the 13th International Histocompatibility Working Group (IHWG) and were used to test the KIR genotyping method developed in this study.

For preeclampsia application, DNA was obtained from the blood samples of 7 pregnant women with preeclampsia and 13 matched women who had normal pregnancies. Cord blood samples from their babies and mouth swabs from their husbands were obtained for genomic DNA isolation. The women were recruited from Seoul National University Hospital (H-9712-038-002).

II. Totalplex amplification of KIR genes

16 KIR genes were amplified by totalplex amplification, a multiplex PCR using SBS primers. SBS primers were designed to anneal at 60℃ and contain 20–27 nucleotides complementary to KIR genes and the central bulge

region (Figure 3, Table 1). KIR gene sequences were referenced in the IPD-KIR database 2.3.0 (http://www.ebi.ac.uk/ipd/kir/) (Robinson *et al.*, 2005). Most of the amplicons of the 16 KIR genes were targeted in exon5. But those of 2DL1, 2DP1, 2DS1, 2DS4 and 3DS1 were targeted in exon4 while the 2DL3 gene was amplified in exon 9.

Genomic DNA from cell lines, human buccal samples, peripheral blood samples and the NK/KIR reference panel I was used for this study. Genomic DNA was amplified with three primer sets (Table 1) in two separate tubes under the following conditions. Exon5 amplification was performed in a 20 μl mixture containing 5 ng of genomic DNA, SBS primer set (0.5 μM of exon 5F, 0.5 μM of exon 5R and 0.1 μM of exon 5R'), 250 μM dNTP mix, 75 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 75 mM KCl, 30 mM $(NH_4)_2SO_4$, and 1.5 U of NeoTaq DNA polymerase (Komabiotech, Seoul, Korea). The primer Exon5 R' was specially designed for the 3DP1 gene, which has a shorter 3'sequence in exon 5 compared to other KIR genes. The reactions were preheated at 94℃ for 5min, and then 35 amplification cycles were carried out in a Master-cycler (Eppendorf, Hamburg, Germany) using the following parameters: denaturation at 94℃ for 20 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 1 min. For exon 4 and exon 9, a primer set of 0.5 μM of exon 4F, 0.5 μM of exon 4R, 0.125 μM of exon 9F and 0.125 μM of exon 9R was used and they were co-amplified under the same conditions as the exon5 amplification. Amplified products were analyzed by electrophoresis on a 1.5% agarose gel run with ethidium bromide staining.

III. GSPE

To analyze each KIR gene amplicon from the mixture of all the amplification products, GSPE was performed. GSPE primers were designed to overcome sequence similarity in KIR genes and distinguish each KIR gene amplicon by a single base difference (Figure 4 and Figure 5A). Table 2 shows the sequence of GSPE primers, which contain a 5' tag sequence specific to each KIR gene. These primers were synthesized, followed by high quality purification. Before being used for GSPE, totalplex amplification products of exon 5 and exon4/9 were mixed and dNTPs remaining in the reaction mixture were removed as follows. 4 μl of the exon 5 PCR products and 2 μl of the exon 4 and exon 9 PCR products were combined, and 2 μl of 1 U antartic phosphatase (New England Biolabs; Ipswich, MA. USA) were added to the mixture. The mixture was incubated at 37℃ for 90 min and 85℃ for 15 min. Then, 8 μl of the activated mixture was added to 12 μl of the GSPE reaction mixture to specifically label each KIR amplicon with biotin-dCTP. The GSPE reaction mixture contained 75 mM Tris-HCl (pH 9.0); 2 mM $MgCl_2$; 50 mM KCl; 20 mM $(NH_4)_2SO_4$; 25 nM GSPE primer mix; 6 μM biotin dCTP; 50 μM of dATP, dGTP and dTTP mix; 0.5 μM dCTP; and 1 U of NeoTaq DNA polymerase. After denaturation at 94℃ for 5 min, 35 amplification cycles were carried out using the following parameters: 94℃ for 30 sec, 57℃ for 1 min and 72℃ for 2 min.

IV. Preparation of beads coupled with probe oligonucleotides

Beads of 16 types were prepared to bind specifically to each KIR GSPE product. Specific binding is mediated by base-pairing between a 5' tag sequence of biotin-labeled KIR amplicon and an anti-tag sequence of bead-coupled oligonucleotide probe. All oligonucleotide probes are composed of 5'-end amine modification for bead coupling, 15 bp oligodT sequences for hybridization flexibility, and anti-tag sequences specific for each KIR gene (Table 3). The synthesized probes were coupled to carboxylated beads (Luminex Corp; Austin, TX, USA) through a carbodiimide base coupling procedure. The beads are 5.6 μm polystyrene microspheres that are internally dyed with two spectrally distinct fluorochromes (red and infrared) (Figure 5B). Using precise amounts of each of these fluorochromes, an array is created consisting of 100 different microsphere sets with specific spectral addresses. For the coupling of probe oligonucleotides to the beads, approximately 480,000 carboxylated beads were suspended in 20 μl of 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (pH 4.5), and then 400 pmol of probe oligonucleotides and 200 μg of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbo-diimide (EDC) were added and mixed thoroughly. Incubation was carried out in the dark under agitation for 30 min and repeat agitation for 20 min with additional 200 μg EDC. Then the beads were washed sequentially with 0.5 mL of 0.02% Tween 20. 0.5 ml of 0.1% of sodium dodecyl sulfate before being stored in 200 μl of TE buffer at 4℃ in the dark.

V. Data collection using bead array

The types of amplified KIR genes can be identified by hybridizing the tag sequence of the biotin-labeled KIR amplicon with the anti-tag sequence on the beads. The hybridization reaction of biotin-labeled GSPE products to microsphere bead was followed by manufacture's protocols with minor modification (Oh *et al.*, 2007). Briefly, 1 μl of bead mixture (16 different bead types, one thousand microspheres each) and 20 μl of 2x hybridization solution (YeBT, Seoul, Korea) were added to the 20 μl of biotin labeled GSPE products. The mixtures were denatured for 10 min at 95℃ and then incubated for 30 min at 37℃. The hybridized microspheres were washed three times in 160 μl of washing buffer, containing 0.2 M NaCl, 0.1 M Tris (pH 8.0) and 0.08% Triton X-100. Streptavidin–R-phycoerythrin (MOSS; Pasadena, MD, USA) was diluted (1:500) in washing buffer, and of which 100 μl was added to the hybridized microsphere (Figure 6A). The mixture was then shaken in 250 rpm for 15 min at room temperature for fluorescence labeling. These reactions were carried out in a96-well plate.

Microsphere fluorescence was then measured using a Luminex 200 cytometer (Luminex; Austin, TX, USA) equipped with a Luminex XYP plate reader and Masterplex GT software (Miraibio; San Francisco, CA, USA). Microspheres are interrogated individually in a rapidly flowing fluid stream as they pass by two separate lasers in the Luminex 200^{TM} analyzer. A 635 nm, 10 mW red diode laser excites the two fluorochromes contained within the microspheres and a 532 nm, 13 mW yttrium aluminum garnet (YAG) laser excites the reporter fluorochrome (R-phycoerythrin) bound to the microsphere

surface. High-speed digital signal processing classifies the microsphere based on its spectral address and quantifies the reaction on the surface. Data were collected from a minimum of 100 microsphere of each type. The median fluorescence intensity (MFI) of any signals that is greater than 600 was considered as the presence of KIR genes. This cut off value for the minimal signal was calculated by the average of the negative MFI value of the 16 types of KIR genes plus 3 standard deviations (SD) (Aslanian *et al.*, 2007).

VI. Validation of totalplex/bead array-based KIR genotyping method

For validation of the totalplex/bead array-based KIR genotyping method, IHWG KIR reference panel I DNA samples were purchased of which 10ng DNA was used in each totalplex amplification. In addition, 17 peripheral blood and 8 buccal samples, which were obtained from Seoul National University Hospital, and 8 human cell lines were used for totalplex/bead array-based KIR genotyping. The results were then compared with those obtained using a SSP-PCR KIR typing kit (MiltenyiBiotec; Auburn, CA USA). The KIR genotyping results using the SSP-PCR kit were kindly provided by Dr. Kang HJ at Seoul National University Hospital.

VII. HLA-C typing

The HLA-C genotyping was performed by SSP-PCR. Because the KIR receptors recognize separately HLA-C1 and HLA-C2, the HLA-C genotype was defined in terms of group C1 or C2. Mouth swabs from the father and the cord blood from the fetus were used for preparation of the genomic DNA. To verify PCR results, two PCR reactions were performed using two different primer pairs (forward-reverse 1, forward-reverse 2) to amplify HLA-C. The DRB1 gene was amplified in the same reaction mixture. The nucleotide sequences for primers are listed in Table 4. Amplification was performed in a 20 μl mixture containing 50 ng of genomic DNA, 200 μM dNTPs, 1 μM HLA-C primers with 350 nM DRB primers and 1.5 U of NeoTaq DNA polymerase. The PCR reactions were performed in an Applied Biosystems Veriti 96-well thermal cycler (Calsbad, CA, USA) using the following program: 96℃, 1 min; 4 cycles of [96℃, 25 sec; 70℃, 45 sec; 72℃, 30 sec]; 24 cycles of [96℃, 25 sec; 65℃, 45 sec; 72℃, 30 sec]; 5 cycles of [96℃, 25 sec; 58℃, 1 min; 72℃, 2 min]; and 72℃, 10 min.

RESULTS

I. Development of totalplex/bead array-based KIR genotyping method

The whole procedure for totalplex/bead array-based KIR genotyping is comprised of the following four steps; 1) primary gene amplification using SBS primers, 2) activation and labeling of products with biotin-dCTP, 3) hybridization of labeled products to bead mixture and 4) reading and analysis using a Luminex 200 cytometer. This is summarized in Figure 7.

Difficulty in KIR genotyping is due to the 85–99% sequence similarity between the 16 KIR genes even though each allele has diverse segments. In this study, I overcame the difficulties in the sequence difference using totalplex amplification. Instead of using 16 gene-specific primer sets, three sets of primers were applied to amplify all the 16 KIR genes (Figure 8). Most of the KIR genes were amplified in exon5 with SBS primers; 2DL1, 2DP1, 2DS1, 2DS4 and 3DS1 in exon 4; and 2DL3 in exon 9 (Figure 8A). The agarose gel run of exon 5 PCR products showed that 380 bp amplicons of exon 5 were observed in all DNA samples examined (Figure 8B, upper panel). In addition, amplicons of exon 4 (300 bp) and exon 9 (162 bp) were observed in all samples (Figure 8B, lower panel). SBS primers for exon 5 were used to amplify 10 KIR genes at the same time with high specificity. A characteristic feature of SBS primers comes from the central bulge region, which structurally separates 3'-end and 5'-end segments. As four consecutive dTTPs

were designed to correspond to any two nucleotides in the target sequence, a solid bulge structure will form in the center of the SBS primer when annealed to the target sequence. The bulge part works as a hinge during the hybridization process (Kang *et al.*, 2008). This hinge makes the SBS primer select one of the three possible initial hybridization mechanisms; hybridize to 3'-end segments, hybridize to 5'-end segments or hybridize simultaneously to 3'-end and 5'-end segments. Because all three possible mechanisms have different Tm values, the SBS primer could have a broad annealing temperature. In the late stage of the amplification cycles, four consecutive dTMPs in bulge are amplified to four consecutive dAMPs in the new template and no bulge is made in the SBS primer. As a result, the long SBS primer without the bulge has a high annealing temperature and very high specificity as the cycles continue. Therefore, the SBS primer can cover the different sequences of primer binding sites. Then, I solved the problem of sequence similarity in KIR genes using GSPE. GSPE primers contain unique single base only present in one KIR gene at its 3' end. When the GSPE primer binds to other KIR amplicons, the primer extension cannot go ahead because of the single base mismatch on the 3' end. Accordingly, the 16 KIR genes were differentiated and labeled with GSPE primer. Finally, I used the bead array system (Luminex), as it can detect 100 different targets so that differentially labeled KIR genotypes can be analyzed in one well. Totalplex/bead array-based KIR genotyping results using the Beas2b cell line and one human buccal swab sample are presented in Figure 9 and Figure 10, respectively. The results demonstrate that the presence or absence of specific KIR genes can be

clearly judged from MFI values. The positive signal (MFI>600) showed at least a 10 times higher MFI value than the negative signal (MFI<600).

II. Validation of totalplex/bead array-based KIR genotyping results

To test the accuracy of totalplex/bead array-based KIR genotyping, I performed KIR genotyping using a total of 48 samples of NK/KIR reference panel I obtained from IHWG. The KIR genotyping results coincide with the IHWG reference typing information (Table 5).

Next, I determined KIR genotypes of 8 human cell lines (Table 6), 8 human buccal samples (Table 7), and 17 peripheral blood samples (Table 8), and then compared the results with those obtained by SSP-PCR. Totalplex/bead array-based KIR genotyping results demonstrated a 100% coincidence with those from SSP-PCR in all the samples examined. These findings suggest that totalplex/bead array-based KIR genotyping is a useful method for the diagnosis of the KIR genotype in a clinical setting.

Because 2DL4, 3DL2, 2DL3 and 3DP1 are structure genes (framework genes, Figure 1) that are common in both haplotypes, they were detected in all the samples examined. In addition, 2DS4 and 2DP1 KIR genotypes were predominantly observed in all the primary samples examined.

III. Reproducibility of totalplex/bead array-based KIR genotyping

To confirm the reproducibility of the newly developed KIR genotyping method, the same samples were reanalyzed 6 times and the results were compared (Figure 11). Genomic DNA samples prepared from three cell lines (HeLa, 293, SHSY5Y) and one peripheral blood sample were used. The results showed that the KIR genotype of each sample was reproducibly verified and that the MFI of each KIR gene in the same DNA sample turned out similar in every repetitive experiment. These results indicate that a newly developed KIR genotyping method is stable and reproducible.

IV. Detection sensitivity of totalplex/bead array-based KIR genotyping

To determine the detection limits of totalplex/bead array-based KIR genotyping, the genomic DNA of three cell lines and one peripheral blood sample was used (Table 9). The serial dilutions of the samples (1 ng, 2 ng, 5 ng, 10 ng and 100 ng per reaction) were used for totalplex/bead array-based KIR genotyping. The results showed a significant relationship between the amount of template and the signal intensities (data not shown). The KIR typing using 1 ng and 2 ng of DNA resulted in false results (gray shadow in Table 9). However, when 5 ng or a higher amount of genomic DNA was used, KIR genotyping results were stable. These results suggest that the

totalplex/bead array-based assay is highly sensitive.

V. KIR genotyping application to preeclampsia patients

I applied the newly developed KIR genotyping method to the prediction of preeclampsia. A total 13 normal pregnant families (women, her male partner and fetus) and 7 preeclampsia families were tested. Among the preeclampsia families, fetuses in two families were lethal and cord blood samples could not be obtained. KIR genotyping results of all the pregnant women and their fetuses are shown in Table 10. Among the 20 families, 11 mother-fetus pairs had the same KIR genotype. KIR haplotypes of normal pregnant women were almost the same as those of their fetus. However, in the preeclampsia group, just one pair had the same haplotypes, AA (Table 11). To confirm the effect of inherited HLA-C2 on preeclampsia development, I analyzed HLA-C genotypes of fathers and fetuses (Table 11). All the fetuses examined shared at least one HLA-C haplotype with their father, confirming that the father's HLA-C haplotype had been inherited by his offspring. Among the preeclampsia families, only the PE2 family demonstrated a match with the maternal KIR AA haplotypes and fetal HLA-C2/C2 haplotypes.

DISCUSSION

The importance of accurate KIR genotyping has been increasing in critical settings. Major improvements in the development of innovative KIR genotyping tests for clinical use have allowed medical professionals to determine and implement the best treatment options for patients. Several nucleic acid-based methods have been utilized to identify and quantify specific KIR types in clinical samples. However, the PCR-based methods have shown variations in amplification efficiencies with relatively low efficiency. In addition, traditional PCR-based methods are single-locus assays that limit clinical application of these methods to KIR genotyping. KIR genes have a high degree of homology from one KIR gene to another, which leads to an excessive rate of false-positive results. In this study, I developed a novel KIR genotyping method which uses totalplex amplification and bead array. Because multiple fluorophores were used, a number of KIR genes could be detected simultaneously in a single PCR reaction tube. In addition, this technique proved to be highly sensitive and reproducible.

There are two major phenomena that may skew the template to product ratio in multiplex PCR: PCR selection and PCR drift (Polz and Cavanaugh, 1998). The use of primers with high binding efficiency (high CG-content) may lead to PCR selection and stochastic variation in the early cycles of the reaction, which causes PCR drift. Our results suggest that SBS primers overcome the bias problem in multiplex PCR because of their unique nature. The bulge sequence comprised of the dTTP homopolymer separates the 3’ and 5’ segments, which bind to their corresponding templates depending on the

Tm or GC content of each segment. The SBS primer is stabilized before extension as both end regions bind to the template. Thus, the SBS primer can have three different initial annealing temperatures; the 5' end segment, 3'end segment and overall primer annealing temperatures, resulting in a broader annealing temperature range for primer and template. The two short 5' and 3' end segment sequences (12 - 20 bp each) have a lower annealing temperature than the whole SBS primer. Mismatches can be avoided by using an annealing temperature that is higher than the two short segment annealing temperatures. The lower annealing temperature of the 5' and 3' end segments will confer higher specificity when the entire SBS primer is stabilized on the template before extension. In conclusion, the bulge structure of the SBS primer may extend the annealing temperature range and increase the fidelity of primer and template binding. The specificity of the newly developed method was verified using the IHWG NK/KIR reference panel. KIR genotyping results were 100% concordant with the IHWG reference typing information. Furthermore, all genomic DNA from the 17 peripheral blood cells, 8 established cell lines and 8 buccal cells were typed for KIR genes with this novel system and showed 100% identical results with SSP-PCR.

Many KIR genotyping methods such as SSP-PCR or sequencing-based methods are not high throughput and require a large amount of DNA template, depending on the typing method (Houtchens *et al*., 2007; Nong *et al*., 2007; Vilches, *et al*., 2007). Most KIR genotyping methods have used sequence-specific primer pairs for each KIR gene. The efficient development of 16 KIR genes typing requires adjustment of the amplifying location, primer Tm, optimized primer combination and reaction conditions for every PCR reaction.

Our system has the best primer mix condition. Totalplex amplification is a simple step that uses only 3 kinds of primer sets, including the SBS primer instead of 16 primer pairs. Subsequent GSPE steps improve accuracy by short length amplification near the single base difference site. Then, the use of the bead array makes this assay a high throughput detection system.

For high throughput KIR genotyping, the SSOP blot method has offered some advantages over SSP because it is amenable to the analysis of hundreds of samples concurrently and requires less DNA (< 1 μg). However, the totalplex/bead array-based KIR genotyping method proves to be more sensitive than any other reported KIR typing methods. Current KIR genotyping methods can use as little as 5 ng of genomic DNA to assign 16 genotypes in a 96-well based assay format. The accuracy greatly declines below 5 ng of genomic DNA. Consuming the minimum amount of sample is important to facilitate clinical and research applications. In conclusion, it has also advantages in sample amount as well as high throughput. This system saves template DNA, labor and reagent costs for typing 16 types of KIR genes genotyping in a one-tube reaction. This method also can provide a rapid and convenient screening assay for the study of KIR gene functions in allogeneic transplantation and other applications.

The newly developed method may be applicable to histocompatibility testing for safe transplantation. KIR and HLA incompatibility may influence the outcome of allogeneic hematopoietic stem-cell transplants. A donor NK-cell repertoire contains some precursors that only have one kind of inhibitory receptor recognizing self MHC ligands. Donor NK cells will be activated in a recipient who does not have correspondent ligands (Ruggeri *et al.*, 2005;

Velardi, 2008; Leung, 2011). Therefore, identification of both HLA and KIR genotypes will decrease the graft-versus-host disease incidence (Parham and McQueen, 2003). In addition to transplantation, previous research has also reported on many KIR-related diseases; 3DL1 and 3DS1 in resistance to HIV attack, correlation of 2DL3 and HLA-C1 in prevention of HCV infection, 2DL2 in rheumatoid arthritis, high expression levels of 2DL1, 2DL2 and 2DL3 in leukemia, and the correlation between HLA-C2 and KIR AA haplotypes in preeclampsia.

Previous reports have shown that couples in which the female partner has the KIR AA haplotypes and in particular lacks KIR2DS1, and the male partner is HLA-C2 homozygous, have the poorer prognosis for preeclampsia than those in which the male partner is HLA-C1 homozygous (Hiby *et al*., 2004; Hiby *et al.*, 2008). Therefore, I examined whether there is a correlation between KIR AA and HLA-C2. The mother's KIR genotype will shape the response of uterine NK cells and, in addition, she will also donate an HLA-C2 gene to the fetus. In addition, the fetus will inherit the HLA genes from the father. Therefore, the mother's KIR genotype and fetus's HLA type are important to preeclampsia. KIR2DS1 is the activating receptor for HLA-C2 groups and so the functional effect would be to overcome the strong inhibition mediated by a HLA-C2-KIR2DL1 interaction (Stewart *et al.*, 2005). For women carrying an HLA-C2-expressing fetus, the risk of preeclampsia is reduced by the presence of a group B KIR haplotype to an extent that correlates with the total number of activating KIR genes. Thus, the effects of HLA-C2-mediated inhibition are offset by the presence of activating KIR genes. The weak interaction of KIR2DS1 with HLA-C2 might generate

activating signals that mute the inhibitory signals from KIR2DL1. For the other activating receptors (that is, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1), the ligands are unknown, as is how they might exert their effects to reduce preeclampsia. In a different study, women who have experienced recurrent spontaneous abortion were reported to have fewer inhibitory KIR genes on average than women in the control group (Varla-Leftherioti *et al.*, 2003). In this study, among the seven preeclampsia families, only one mother-fetus showed KIR AA and HLA-C2. It has been reported that there is ethnic variation in the frequencies of KIR AA and HLA-C2 types (Moffett *et al*., 2006). In Asians, the KIR AA haplotype is over 50% whereas the HLA-C2 homozygote is lower than 20%. Because of the small number, I could not demonstrate clearly the correlation between the presence of a KIR AA/HLA-C2 homozygote match and preeclampsia risk, necessitating further research.

CONCLUSION

Molecular diagnostics utilizing the newest molecular biological techniques have been increasingly applied to identify the underlying molecular defects in inherited disease, detect pathogens and analyze traits from the differences in genetic sequence variations. In this study, a totalplex method with special primers with broad Tm values was developed. Then, simultaneous sequence analysis systems using GSPE and Luminex bead array systems were employed for high throughput analysis. The newly developed KIR genotyping method showed high sensitivity and reproducibility, therefore can be effectively applied in several molecular diagnostic fields.

REFERENCES

Ahlenstiel, G., Martin, M. P., Gao, X., Carrington, M. and Rehermann, B. (2008) Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. *J Clin Invest* **118**, 1017-1026.

Aldrich, C., Verp, M. S., Walker, M. A. and Ober, C. (2000) A null mutation in HLA-G is not associated with preeclampsia or intrauterine growth retardation. *J Reprod Immunol* **47**, 41-48.

Anderson, U. D., Olsson, M. G., Kristensen, K. H., Akerstrom, B. and Hansson, S. R. (2011) Review: Biochemical markers to predict preeclampsia. *Placenta* **33 Suppl**, S42-47.

Anfossi, N., Andre, P., Guia, S., Falk, C. S., Roetynck, S., Stewart, C. A., Breso, V., Frassati, C., Reviron, D., Middleton, D., Romagne, F., Ugolini, S. and Vivier, E. (2006) Human NK cell education by inhibitory receptors for MHC class I. *Immunity* **25**, 331-342.

Ashouri, E., Ghaderi, A., Reed, E. F. and Rajalingam, R. (2009) A novel duplex SSP-PCR typing method for KIR gene profiling. *Tissue Antigens* **74**, 62-67.

Aslanian, S., Azimi, M., Noble, J. and Hoppe, C. (2007) Application of flow

cytometry-based genotyping for rapid detection of hemoglobin variants. *Int J Lab Hematol* **29**, 284-291.

Bancroft, G. J. (1993) The role of natural killer cells in innate resistance to infection. *Curr Opin Immunol* **5**, 503-510.

Bao, X. J., Hou, L. H., Sun, A. N., Qiu, Q. C., Yuan, X. N., Chen, M. H., Chen, Z. X. and He, J. (2010) The impact of KIR2DS4 alleles and the expression of KIR in the development of acute GVHD after unrelated allogeneic hematopoietic SCT. *Bone Marrow Transplant* **45**, 1435-1441.

Carrington, M. and Martin, M. P. (2006) The impact of variation at the KIR gene cluster on human disease. *Curr Top Microbiol Immunol* **298**, 225-257.

Cooper, M. A., Elliott, J. M., Keyel, P. A., Yang, L., Carrero, J. A. and Yokoyama, W. M. (2009) Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci U S A* **106**, 1915-1919.

Crum, K. A., Logue, S. E., Curran, M. D. and Middleton, D. (2000) Development of a PCR-SSOP approach capable of defining the natural killer cell inhibitory receptor (KIR) gene sequence repertoires. *Tissue Antigens* **56**, 313-326.

Edwards, M. C. and Gibbs, R. A. (1994) Multiplex PCR: advantages, development, and applications. *PCR Methods Appl* **3**, S65-75.

Furukawa, H., Yabe, T., Watanabe, K., Miyamoto, R., Miki, A., Akaza, T., Tadokoro, K., Tohma, S., Inoue, T., Yamamoto, K. and Juji, T. (1999) Tolerance of NK and LAK activity for HLA class I-deficient targets in a TAP1-deficient patient (bare lymphocyte syndrome type I). *Hum Immunol* **60**, 32-40.

Hiby, S. E., Regan, L., Lo, W., Farrell, L., Carrington, M. and Moffett, A. (2008) Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. *Hum Reprod* **23**, 972-976.

Hiby, S. E., Walker, J. J., O'Shaughnessy K, M., Redman, C. W., Carrington, M., Trowsdale, J. and Moffett, A. (2004) Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* **200**, 957-965.

Houtchens, K. A., Nichols, R. J., Ladner, M. B., Boal, H. E., Sollars, C., Geraghty, D. E., Davis, L. M., Parham, P. and Trachtenberg, E. A. (2007) High-throughput killer cell immunoglobulin-like receptor genotyping by MALDI-TOF mass spectrometry with discovery of novel alleles. *Immunogenetics* **59**, 525-537.

Hsu, K. C., Chida, S., Geraghty, D. E. and Dupont, B. (2002a) The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev* **190**, 40-52.

Hsu, K. C., Liu, X. R., Selvakumar, A., Mickelson, E., O'Reilly, R. J. and Dupont, B. (2002b) Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol* **169**, 5118-5129.

Huard, B. and Fruh, K. (2000) A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells. *Eur J Immunol* **30**, 509-515.

Jebbink, J., Wolters, A., Fernando, F., Afink, G., van der Post, J. and Ris-Stalpers, C. (2012) Molecular genetics of preeclampsia and HELLP syndrome - A review. *Biochim Biophys Acta* **1822**, 1960-1969.

Johansson, S., Berg, L., Hall, H. and Hoglund, P. (2005) NK cells: elusive players in autoimmunity. *Trends Immunol* **26**, 613-618.

Kang, H. J., Oh, Y., Chun, S. M., Seo, Y. J., Shin, H. Y., Kim, C. W., Ahn, H. S. and Han, B. D. (2008) TotalPlex gene amplification using bulging primers for pharmacogenetic analysis of acute lymphoblastic leukemia. *Mol Cell Probes* **22**, 193-200.

Karre, K., Ljunggren, H. G., Piontek, G. and Kiessling, R. (2005) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. 1986. *J Immunol* **174**, 6566-6569.

Khakoo, S. I. and Carrington, M. (2006) KIR and disease: a model system or system of models? *Immunol Rev* **214**, 186-201.

Kikuchi-Maki, A., Catina, T. L. and Campbell, K. S. (2005) Cutting edge: KIR2DL4 transduces signals into human NK cells through association with the Fc receptor gamma protein. *J Immunol* **174**, 3859-3863.

Kulkarni, S., Martin, M. P. and Carrington, M. (2008) The Yin and Yang of HLA and KIR in human disease. *Semin Immunol* **20**, 343-352.

Kulkarni, S., Martin, M. P. and Carrington, M. (2009) KIR genotyping by multiplex PCR-SSP. *Methods Mol Biol* **612**, 365-375.

Larroque, B., Breart, G., Kaminski, M., Dehan, M., Andre, M., Burguet, A., Grandjean, H., Ledesert, B., Leveque, C., Maillard, F., Matis, J., Roze, J. C. and Truffert, P. (2004) Survival of very preterm infants: Epipage, a population based cohort study. *Arch Dis Child Fetal Neonatal Ed* **89**, F139-144.

Leung, W. (2011) Use of NK cell activity in cure by transplant. *Br J Haematol* **155**, 14-29.

Liao, N. S., Bix, M., Zijlstra, M., Jaenisch, R. and Raulet, D. (1991) MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* **253**, 199-202.

Ljunggren, H. G., Stam, N. J., Ohlen, C., Neefjes, J. J., Hoglund, P., Heemels, M. T., Bastin, J., Schumacher, T. N., Townsend, A., Karre, K. and et al. (1990) Empty MHC class I molecules come out in the cold. *Nature* **346**, 476-480.

Long, E. O. (1999) Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol* **17**, 875-904.

Lopez-Botet, M., Llano, M. and Ortega, M. (2001) Human cytomegalovirus and natural killer-mediated surveillance of HLA class I expression: a paradigm of host-pathogen adaptation. *Immunol Rev* **181**, 193-202.

Maconochie, N., Doyle, P., Prior, S. and Simmons, R. (2007) Risk factors for first trimester miscarriage--results from a UK-population-based case-control study. *BJOG* **114**, 170-186.

Marsh, S. G. (2004) Nomenclature for factors of the HLA system, update May 2004. *Hum Immunol* **65**, 1546-1547.

Meekins, J. W., Pijnenborg, R., Hanssens, M., McFadyen, I. R. and van Asshe,

A. (1994) A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *BJOG* **101**, 669-674.

Meenagh, A., Gonzalez, A., Sleator, C., McQuaid, S. and Middleton, D. (2008) Investigation of killer cell immunoglobulin-like receptor gene diversity, KIR2DL1 and KIR2DS1. *Tissue Antigens* **72**, 383-391.

Middleton, D. and Gonzelez, F. (2009) The extensive polymorphism of KIR genes. *Immunology* **129**, 8-19.

Moesta, A. K., Norman, P. J., Yawata, M., Yawata, N., Gleimer, M. and Parham, P. (2008) Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol* **180**, 3969-3979.

Moffett, A., Hiby, S. and Carrington, M. (2006) The incidence of pre-eclampsia among couples consisting of Japanese women and Caucasian men'. *J Reprod Immunol* **71**, 132-133; author reply 134.

Moster, D., Lie, R. T. and Markestad, T. (2008) Long-term medical and social consequences of preterm birth. *N Engl J Med* **359**, 262-273.

Nong, T., Saito, K., Blair, L., Tarsitani, C. and Lee, J. H. (2007) KIR genotyping by reverse sequence-specific oligonucleotide

methodology. *Tissue Antigens* **69 Suppl 1**, 92-95.

O'Leary, J. G., Goodarzi, M., Drayton, D. L. and von Andrian, U. H. (2006) T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* **7**, 507-516.

Oh, Y., Bae, S. M., Kim, Y. W., Choi, H. S., Nam, G. H., Han, S. J., Park, C. H., Cho, Y., Han, B. D. and Ahn, W. S. (2007) Polymerase chain reaction-based fluorescent Luminex assay to detect the presence of human papillomavirus types. *Cancer Sci* **98**, 549-554.

Orr, M. T. and Lanier, L. L. (2010) Natural killer cell education and tolerance. *Cell* **142**, 847-856.

Parham, P. (2004) NK cells and trophoblasts: partners in pregnancy. *J Exp Med* **200**, 951-955.

Parham, P. (2005) MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* **5**, 201-214.

Parham, P. and McQueen, K. L. (2003) Alloreactive killer cells: hindrance and help for haematopoietic transplants. *Nat Rev Immunol* **3**, 108-122.

Polz, M. F. and Cavanaugh, C. M. (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* **64**, 3724-3730.

Rajagopalan, S. and Long, E. O. (2005) Understanding how combinations of HLA and KIR genes influence disease. *J Exp Med* **201**, 1025-1029.

Rajalingam, R., Gardiner, C. M., Canavez, F., Vilches, C. and Parham, P. (2001) Identification of seventeen novel KIR variants: fourteen of them from two non-Caucasian donors. *Tissue Antigens* **57**, 22-31.

Redman, C. W. and Sargent, I. L. (2005) Latest advances in understanding preeclampsia. *Science* **308**, 1592-1594.

Roberts, C. H., Turino, C., Madrigal, J. A. and Marsh, S. G. (2007) Enrichment of individual KIR2DL4 sequences from genomic DNA using long-template PCR and allele-specific hybridization to magnetic bead-bound oligonucleotide probes. *Tissue Antigens* **69**, 597-601.

Roberts, J. M. and Hubel, C. A. (2009) The two stage model of preeclampsia: variations on the theme. *Placenta* **30 Suppl A**, S32-37.

Robinson, J., Waller, M. J., Stoehr, P. and Marsh, S. G. (2005) IPD--the Immuno Polymorphism Database. *Nucleic Acids Res* **33**, D523-526.

Ruggeri, L., Capanni, M., Mancusi, A., Perruccio, K., Burchielli, E., Martelli, M. F. and Velardi, A. (2005) Natural killer cell alloreactivity in

haploidentical hematopoietic stem cell transplantation. *Int J Hematol* **81**, 13-17.

Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W. D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., Martelli, M. F. and Velardi, A. (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **295**, 2097-2100.

Sambrook, J. G., Bashirova, A., Andersen, H., Piatak, M., Vernikos, G. S., Coggill, P., Lifson, J. D., Carrington, M. and Beck, S. (2006) Identification of the ancestral killer immunoglobulin-like receptor gene in primates. *BMC Genomics* **7**, 209.

Schaffer, M., Aldener-Cannava, A., Remberger, M., Ringden, O. and Olerup, O. (2003) Roles of HLA-B, HLA-C and HLA-DPA1 incompatibilities in the outcome of unrelated stem-cell transplantation. *Tissue Antigens* **62**, 243-250.

Shiina, T., Inoko, H. and Kulski, J. K. (2004) An update of the HLA genomic region, locus information and disease associations: 2004. *Tissue Antigens* **64**, 631-649.

Shilling, H. G., Lienert-Weidenbach, K., Valiante, N. M., Uhrberg, M. and Parham, P. (1998) Evidence for recombination as a mechanism for KIR diversification. *Immunogenetics* **48**, 413-416.

Sibai, B., Dekker, G. and Kupferminc, M. (2005) Pre-eclampsia. *Lancet* **365**, 785-799.

Single, R. M., Martin, M. P., Gao, X., Meyer, D., Yeager, M., Kidd, J. R., Kidd, K. K. and Carrington, M. (2007) Global diversity and evidence for coevolution of KIR and HLA. *Nat Genet* **39**, 1114-1119.

Stewart, C. A., Laugier-Anfossi, F., Vely, F., Saulquin, X., Riedmuller, J., Tisserant, A., Gauthier, L., Romagne, F., Ferracci, G., Arosa, F. A., Moretta, A., Sun, P. D., Ugolini, S. and Vivier, E. (2005) Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A* **102**, 13224-13229.

Sun, J. C., Beilke, J. N. and Lanier, L. L. (2009) Adaptive immune features of natural killer cells. *Nature* **457**, 557-561.

Sun, J. Y., Gaidulis, L., Dagis, A., Palmer, J., Rodriguez, R., Miller, M. M., Forman, S. J. and Senitzer, D. (2005) Killer Ig-like receptor (KIR) compatibility plays a role in the prevalence of acute GVHD in unrelated hematopoietic cell transplants for AML. *Bone Marrow Transplant* **36**, 525-530.

Sun, J. Y., Gaidulis, L., Miller, M. M., Goto, R. M., Rodriguez, R., Forman, S.

J. and Senitzer, D. (2004) Development of a multiplex PCR-SSP method for Killer-cell immunoglobulin-like receptor genotyping. *Tissue Antigens* **64**, 462-468.

Trinchieri, G. (1989) Biology of natural killer cells. *Adv Immunol* **47**, 187-376.

Uhrberg, M., Valiante, N. M., Shum, B. P., Shilling, H. G., Lienert-Weidenbach, K., Corliss, B., Tyan, D., Lanier, L. L. and Parham, P. (1997) Human diversity in killer cell inhibitory receptor genes. *Immunity* **7**, 753-763.

Varla-Leftherioti, M., Spyropoulou-Vlachou, M., Niokou, D., Keramitsoglou, T., Darlamitsou, A., Tsekoura, C., Papadimitropoulos, M., Lepage, V., Balafoutas, C. and Stavropoulos-Giokas, C. (2003) Natural killer (NK) cell receptors' repertoire in couples with recurrent spontaneous abortions. *Am J Reprod Immunol* **49**, 183-191.

Vatten, L. J. and Skjaerven, R. (2003) Effects on pregnancy outcome of changing partner between first two births: prospective population study. *BMJ* **327**, 1138.

Velardi, A. (2008) Role of KIRs and KIR ligands in hematopoietic transplantation. *Curr Opin Immunol* **20**, 581-587.

Vilches, C., Castano, J., Gomez-Lozano, N. and Estefania, E. (2007)

Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments. *Tissue Antigens* **70**, 415-422.

Vilches, C., Pando, M. J., Rajalingam, R., Gardiner, C. M. and Parham, P. (2000) Discovery of two novel variants of KIR2DS5 reveals this gene to be a common component of human KIR 'B' haplotypes. *Tissue Antigens* **56**, 453-456.

Vilches, C. and Parham, P. (2002) KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* **20**, 217-251.

Wagtmann, N., Rajagopalan, S., Winter, C. C., Peruzzi, M. and Long, E. O. (1995) Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity* **3**, 801-809.

Whang, D. H., Park, H., Yoon, J. A. and Park, M. H. (2005) Haplotype analysis of killer cell immunoglobulin-like receptor genes in 77 Korean families. *Hum Immunol* **66**, 146-154.

Wilson, M. J., Torkar, M., Haude, A., Milne, S., Jones, T., Sheer, D., Beck, S. and Trowsdale, J. (2000) Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A* **97**, 4778-4783.

Winter, C. C., Gumperz, J. E., Parham, P., Long, E. O. and Wagtmann, N. (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol* **161**, 571-577.

Winter, C. C. and Long, E. O. (1997) A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J Immunol* **158**, 4026-4028.

Witt, C. S., Martin, A. and Christiansen, F. T. (2000) Detection of KIR2DL4 alleles by sequencing and SSCP reveals a common allele with a shortened cytoplasmic tail. *Tissue Antigens* **56**, 248-257.

Yawata, M., Yawata, N., Draghi, M., Little, A. M., Partheniou, F. and Parham, P. (2006) Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *J Exp Med* **203**, 633-645.

Yun, G., Tolar, J., Yerich, A. K., Marsh, S. G., Robinson, J., Noreen, H., Blazar, B. R. and Miller, J. S. (2007) A novel method for KIR-ligand typing by pyrosequencing to predict NK cell alloreactivity. *Clin Immunol* **123**, 272-280.

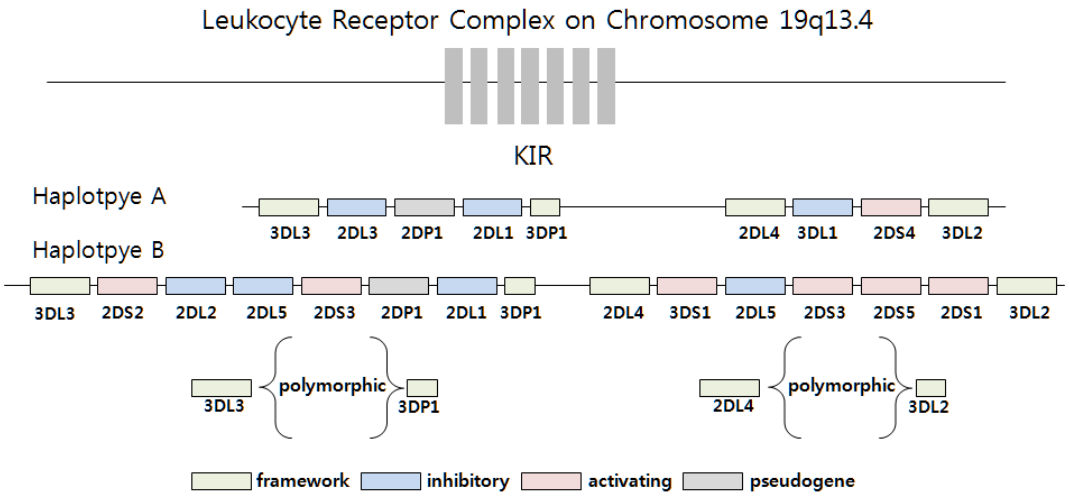


Figure 1. KIR genes organization. The gene family encoding the killer cell immunoglobulin like receptors (KIR) forms part of the leukocyte receptor complex on human chromosome 19q13.4. KIR haplotypes vary extensively in gene content. The A haplotype is fixed in terms of gene content, but the B haplotype is characterized by variable gene numbers.

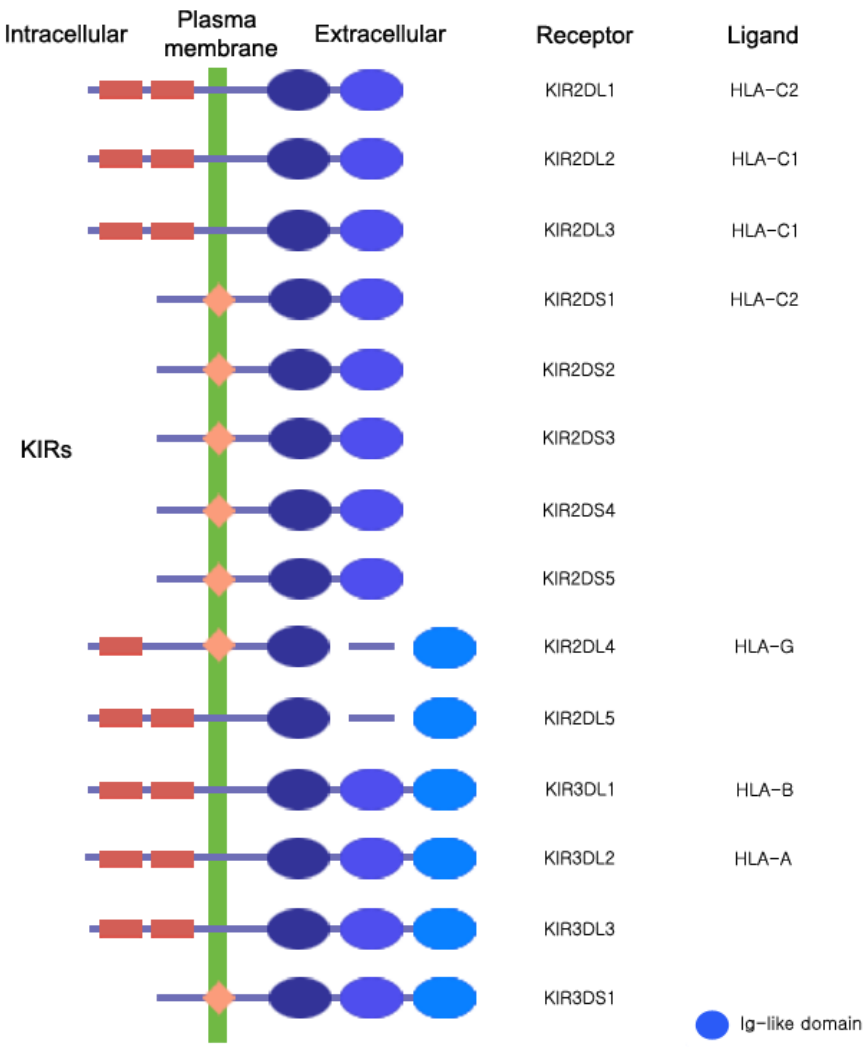


Figure 2. The interactions between KIR and HLA class I. KIRs are a multigene family and its members are often highly polymorphic. KIR genes are named according to the number of extracellular Ig-like domains (2D or 3D) and the size of their cytoplasmic tail, either long (L) or short (S). Each KIR interacts with specific HLA.

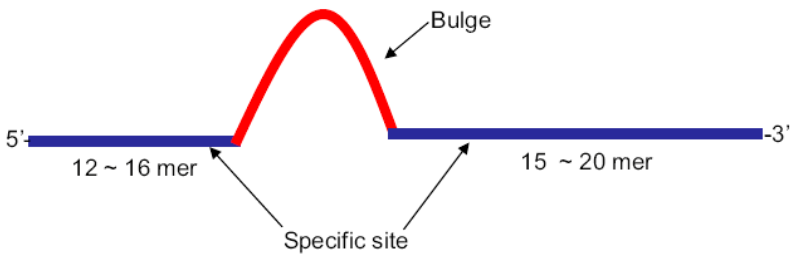


Figure 3. Schematic representation of a specific bulging specific (SBS) primer. The SBS primer is approximately 31 to 40 nucleotides, and comprised of three parts: a 5' end of 12 to 16 nucleotides; a bulge sequence of 4 dTTPs or other deoxynucleotide; and a 3' end of 15 to 20 nucleotides. Bulge region can cover the sequence differences in Exon 5 of each KIR gene, making it possible to amplify different KIR genes at the same time.

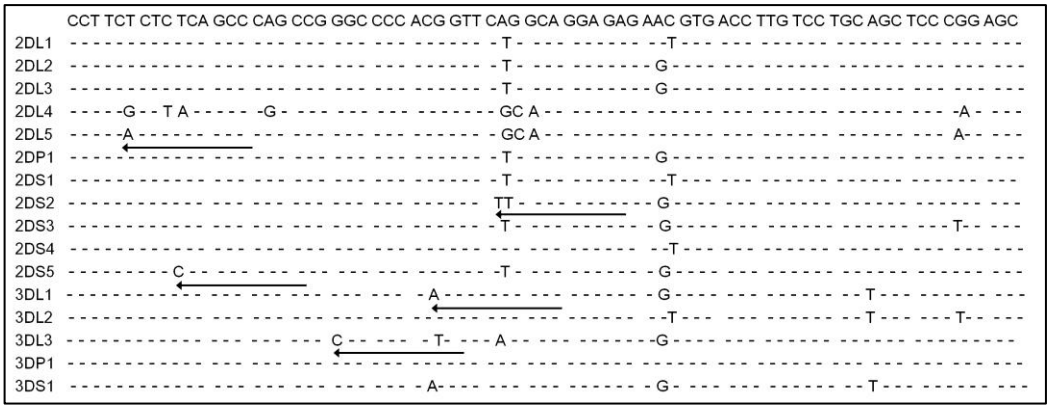


Figure 4. Gene specific primer extension (GSPE) primer design strategy. Each KIR gene displays a high level of sequence similarity. Using single base differences between the KIR genes, GSPE primer is designed that each KIR gene specific nucleotide binds complementarily to the 3' end of primer (black arrow). The sequences are from 108 to 132 in coding sequence.

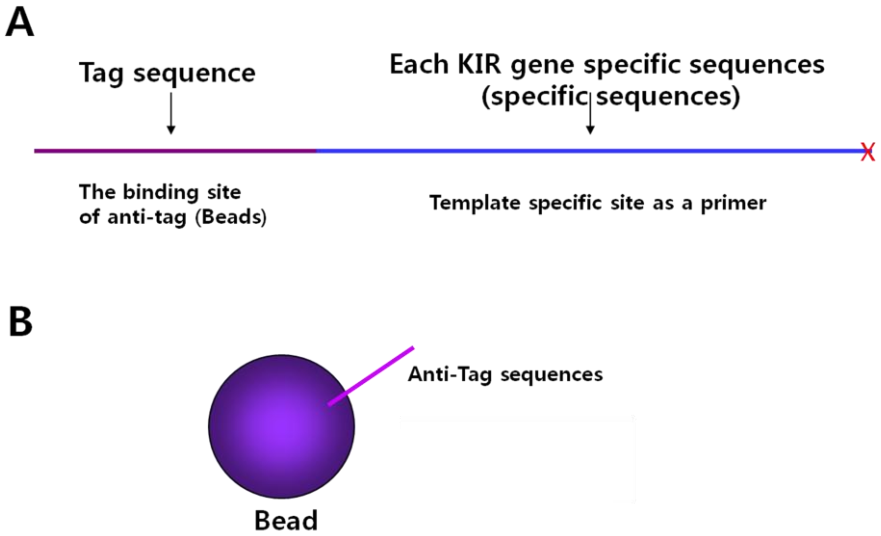


Figure 5. The structure of GSPE primer (A) and probe oligonucleotide-coupled microsphere bead (B). **A.** GSPE primers have tag sequence at 5' end and each KIR gene specific sequence at 3' end which were separated by singe base difference (red X) 3' end. **B.** The beads are 5.6 μm polystyrene microspheres that contain two kinds of dye with spectrally distinct red and infrared and has $5x10^7$ carboxylated binding sites for 5' amine modified oligonucleotide probes which can bind to tag sequence of GSPE primers.

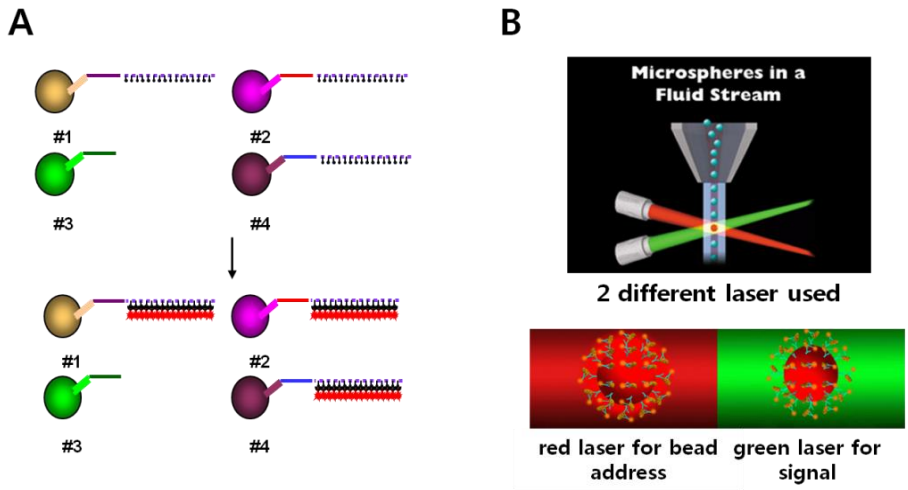


Figure 6. Detection of microsphere fluorescence by two lasers collects the information of spectral address and quantities. **A**. Upper part: hybridization of the tag sequence of biotin-labeled KIR gene to anti-tag sequence on the sixteen different bead types. Lower part: Streptavidin-R-phycoerythrin (SAPE) binds to biotin-labeled KIR amplicons. **B.** Signal acquisition using Luminex. The classification laser (red laser) investigate the dye molecules in each bead by using 635 nm wave length, and the report laser (green laser) investigate the SAPE fluorescence molecules bound on biotin-labeled KIR PCR products at 532 nm.

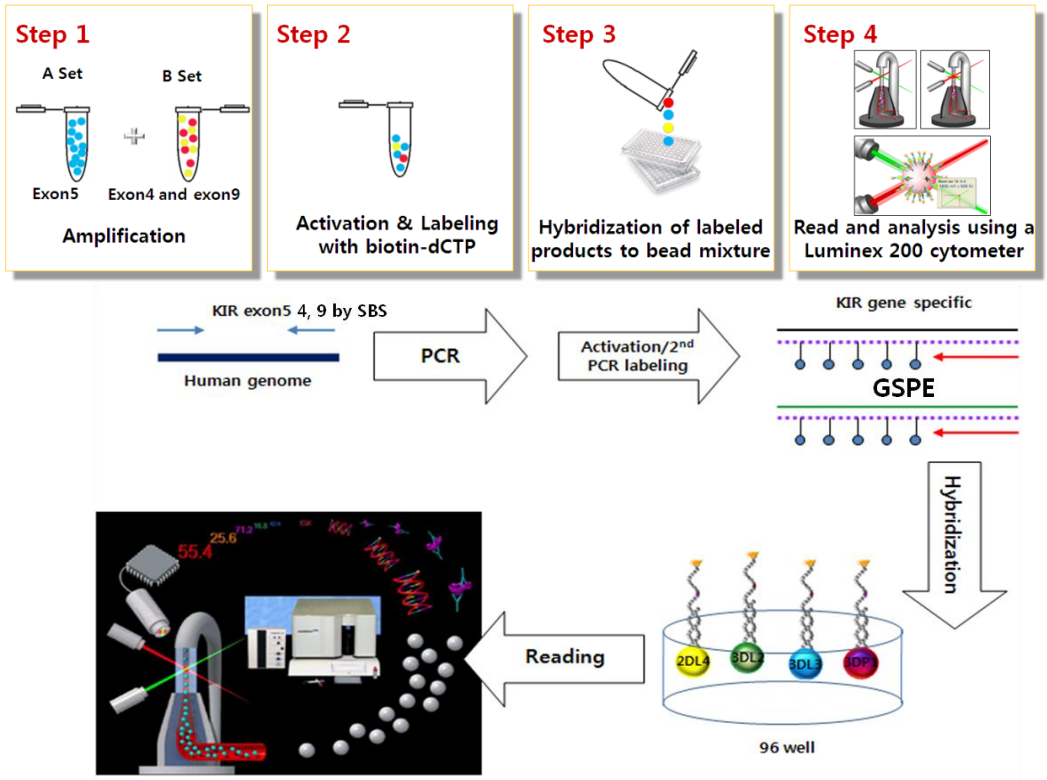


Figure 7. Schematic representation of totalplex/bead array-based KIR genotyping method.

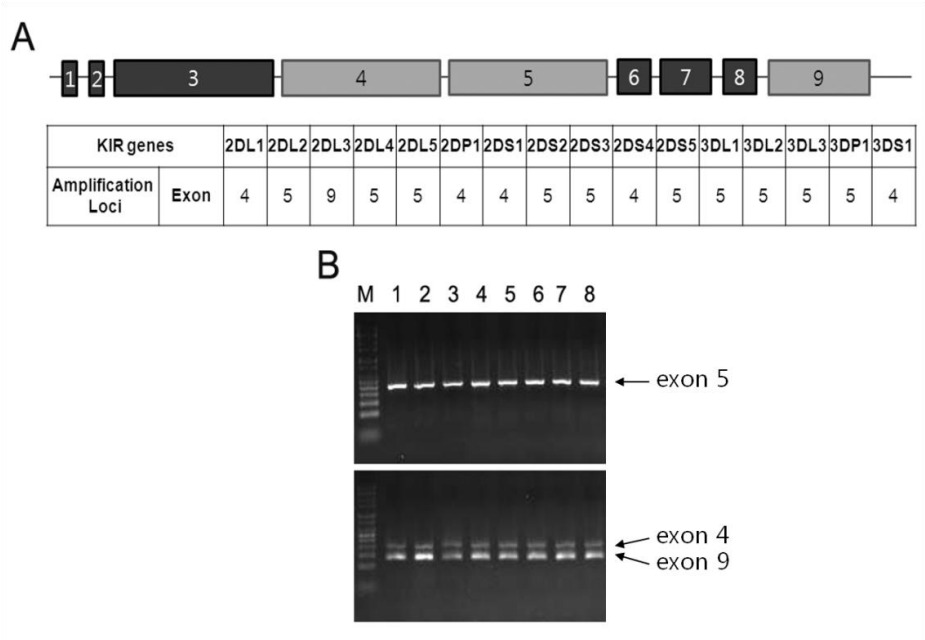


Figure 8. KIR genes amplification loci and the results of totalplex amplification. **A.** Schematic drawing of KIR gene structure (upper part). Lower part specified amplification loci for each KIR gene. **B.** Totalplex amplification products of exon 5 (upper panel) and exon 4/9 (lower panel) were electrophoresed in agarose gel. Amplicon size: exon 5 (380 bp), exon 4 (300 bp), exon 9 (162 bp). Lane 1 to 8: human genomic DNA obtained from buccal samples.

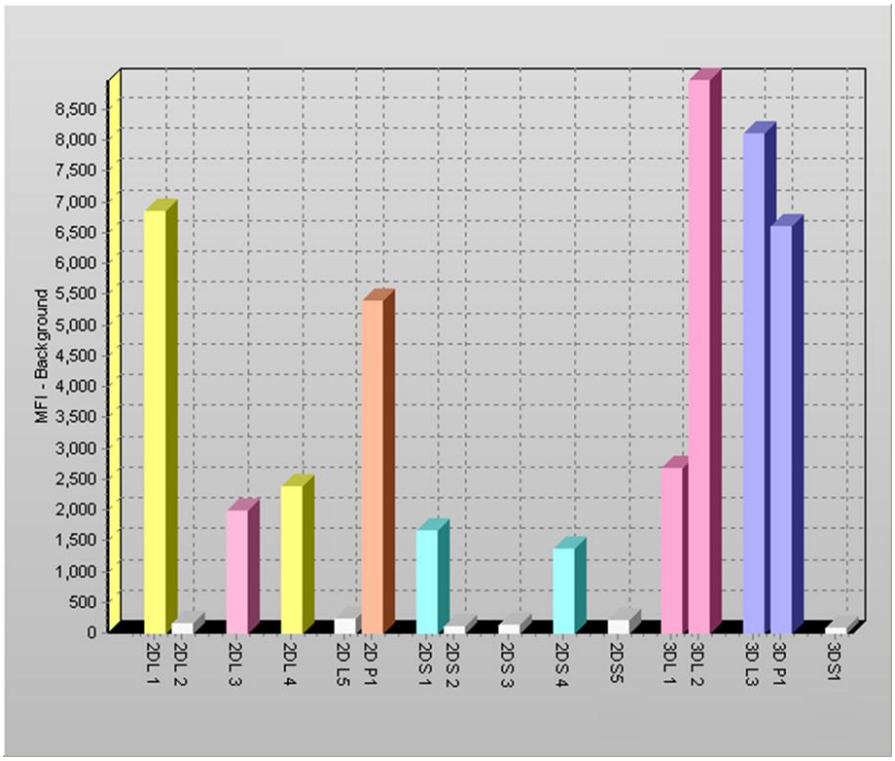


Figure 9. Diagram of KIR genotyping result of a human cell line, Beas2b. The data represent background signal-deduced median fluorescence intensity (MFI). Cutoff value for signal on/off was determined to 600 MFI signal as described in MATERIALS AND METHODS section. Color bar represents the presence of the specific KIR gene while white bar showing the absence of KIR gene.

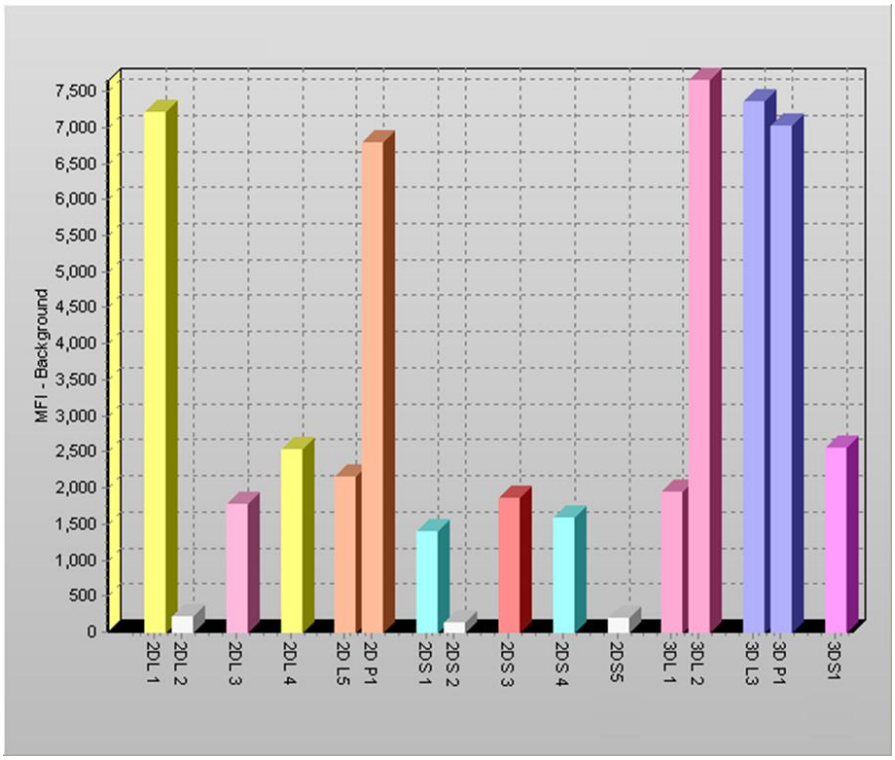
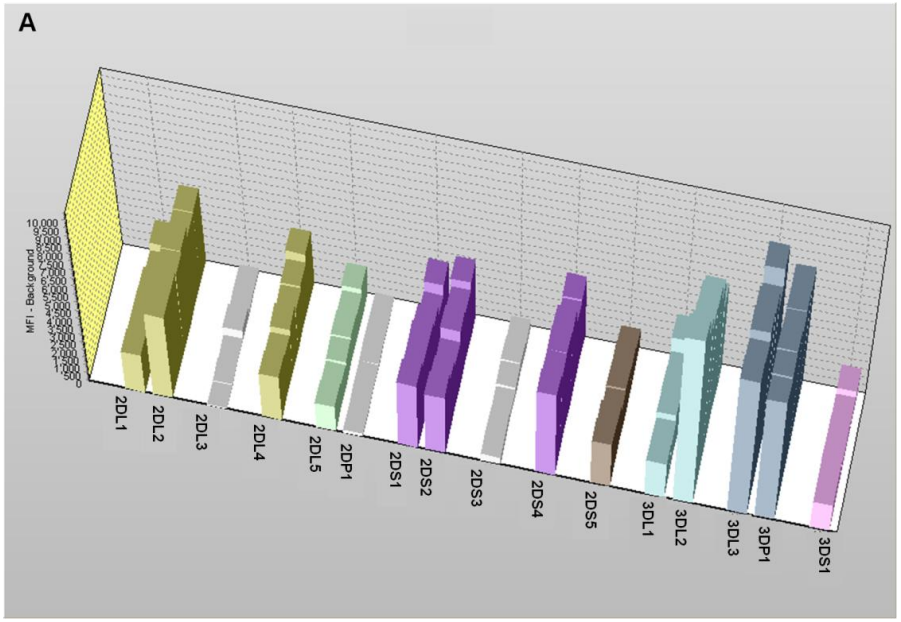
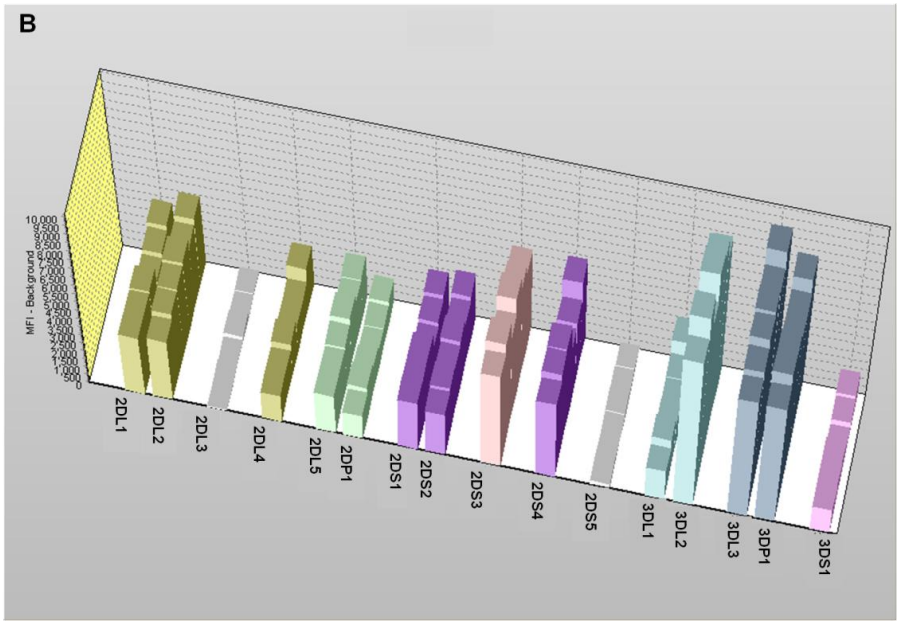


Figure 10. Diagram of KIR genotyping results of a human buccal sample.


A
MFI - Background
10,000
9,500
9,000
8,500
8,000
7,500
7,000
6,500
6,000
5,500
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
2DL1
2DL2
2DL3
2DL4
2DL5
2DP1
2DS1
2DS2
2DS3
2DS4
2DS5
3DL1
3DL2
3DL3
3DP1
3DS1


B
MFI - Background
10,000
9,500
9,000
8,500
8,000
7,500
7,000
6,500
6,000
5,500
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
2DL1
2DL2
2DL3
2DL4
2DL5
2DP1
2DS1
2DS2
2DS3
2DS4
2DS5
3DL1
3DL2
3DL3
3DP1
3DS1

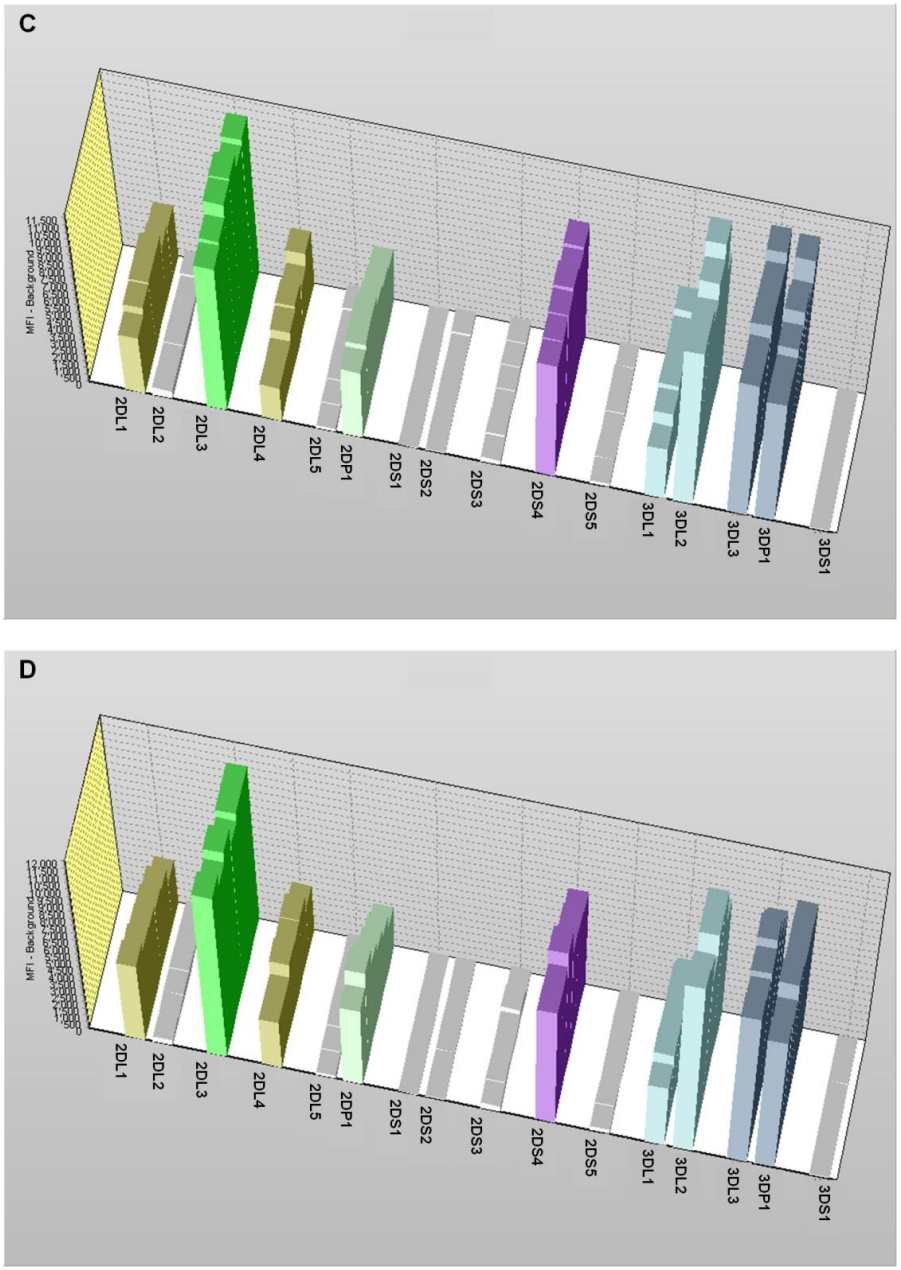


Figure 11. Totalplex/bead array-based KIR genotyping method is highly reproducible. KIR genotyping of 4 different samples was repeated six times and MFI values were compared. A. HeLa cells, B. 293 cells, C. SHSY5Y cells, D. Peripheral blood sample.

Table 1. Primer sequences for totalplex amplification of KIR genes

Primers	Sequences (5' to 3')	Specificity	Location	size(bp)
Exon5 F	AGAGCAGGGGAGTTTTGTTC TCAGCTCAGGT	Exon 5	Intron 4	
Exon5 R	CCTGTGACAGAAACTTTTGC AGTGGGTCACT	Exon 5	287 to 296	380
Exon5 R'	GGAAAGAGCCGAAGTTTTTC T GTAGGTKCCTCC	Exon 5	267 to 277	370
Exon4 F	GTCATCTG CARTGTTGGTC	Exon 4	119 to 125	
Exon4 R	TCCTGGATCATTCACTCTG	Exon 4	Intron 4	300
Exon9 F	AAGACCCTCAGGAGGTGAC	Exon 9	370 to 376	
Exon9 R	CATGGGCAGGAGACAACTTT	Exon 9	418 to 424	162

[+] KIR gene sequences were referred to IPD-KIR database 2.3.0 (16-August-2010, http://www.ebi.ac.uk/ipd/kir)

Table 2. Sequences of primers used for GSPE

Target	Sequences after *tag* (5' to 3')	Location
2DL1	*caatatcatcatctttatcattac* TTCTCCATCAGTCGCATGAC	159 to 165 (Exon 4)
2DL2	*tgatgtttgattatgatgtagtat* GCCCTGCAGAGAACCTACA	243 to 249 (Exon 5)
2DL3	*atactaactcaactaactttaaac* ACTTCCAAATGCTGAGCCCT	410 to 416 (Exon 9)
2DL4	*aatctacaaatccaataatctcat* CCRGGCCGGGCTGTAAGC	204 to 210 (Exon 5)
2DL5	*aatcttactacaaatcctttcttt* CCGGCTGGGCTGAGAGT	204 to 210 (Exon 5)
2DP1	*gatttgtattgattgagattaaag* CATCATGGTGCTCTCCAG	147 to 153 (Exon 4)
2DS1	*cttttacaatacttcaatacaatc* TCCATCAGTCGCATGAA	160 to 165 (Exon 4)
2DS2	*ttacctttatacctttcttttac* TCACGCTCTCTCCTGCCAA	214 to 220 (Exon 5)
2DS3	*tcatcaatcaatctttttcacttt* AGCATCTGTAGGTTCCTCCT	266 to 273 (Exon 5)
2DS4	*atcatacatacatacaaatctaca* CAGTGACCCTCTGGACATG	188 to 194 (Exon 4)
2DS5	*tcataatctcaacaatctttcttt* CCCTCCGTGGGTGGCAGGGT	262 to 268 (Exon 5)
3DL1	*tacatcaacaattcattcaataca* GAGAGAGAAGGTTTCTCATATG	199 to 206 (Exon5)
3DL2	*atgagtatgttattagtgtatgta*	279 to 284 (Exon5)

	TGACCACACGCAGGGCAG	
3DL3	*tcaaaatctcaaatactcaaatca* GGATAGATGGTAAATGTCAAACA	228 to 235 (Exon5)
3DP1	*caattaactacatacaatacatac* TGAGAGAGAAGGTTTCCCAC	200 to 206 (Exon 5)
3DS1	*taattatacatctcatcttctaca* AAGGGCACGCATCATGGA	163 to 168 (Exon 4)

Table 3. Sequences of probe oligonucleotides

Probe type	5’ oligodT	Anti-tag sequences (5' to 3')
2DL1	TTTTTTTTTTTTTT	gtaatgataaagatgatgatattg
2DL2		atactacatcataatcaaacatca
2DL3		gtttaaagttagttgagttagtat
2DL4		atgagattattggatttgtagatt
2DL5		aaagaaaggatttgtagtaagatt
2DP1		ctttaatctcaatcaatacaaatc
2DS1		gattgtattgaagtattgtaaaag
2DS2		gtaaaaagaaaggtataaaggtaa
2DS3		aaagtgaaaaagattgattgatga
2DS4		tgtagatttgtatgtatgtatgat
2DS5		aaagaaagattgttgagattgtga
3DL1		tgtattgaatgaattgttgatgta
3DL2		tacatacactaataacatactcat
3DL3		tgatttgagtatttgagatttga
3DP1		gtatgtattgtatgtagttaattg
3DS1		tgtagaagatgagatgtataatta

Table 4. PCR primer sequences for HLA- C1 and -C2 amplification

Primers	Sequences (5' to 3')	Size (bp)
HLA-C forward	GCCGCGAGTCCRAGAGG	
C1 reverse-1	GCGCAGGTTCCGCAGGC	129
C1 reverse-2	GTTGTAGTAGCCGCGCAGG	141
C2 reverse-1	CGCGCAGTTTCCGCAGGT	130
C2 reverse-2	GTTGTAGTAGCCGCGCAGT	141
DRB forward	AAGACCCTCAGGAGGTGAC	
DRB reverse	GCATCTTGCTCTGTGCAGAT	796

Table 5. Validation of totalplex/bead array-based KIR genotyping results using NK/KIR reference panel I (13th IHWG, consisting of 48 genomic DNAs)

2DL1~ 2DL4	2DL1		2DL2		2DL3		2DL4	
IHW No.	panel	MFI	panel	MFI	panel	MFI	panel	MFI
1003	POS	3386	NEG	370	POS	7316	POS	1912
1010	POS	3344	NEG	312	POS	8304	POS	2137
1016	POS	2830	NEG	222	POS	7883	POS	1410
1017	POS	3267	NEG	333	POS	8490	POS	1905
1018	POS	2285	NEG	273	POS	8811	POS	2132
1021	POS	3483	NEG	222	POS	7724	POS	1770
1029	POS	2927	NEG	307	POS	7084	POS	1827
1031	POS	2915	NEG	331	POS	7719	POS	1924
1040	POS	3092	POS	5074	NEG	64	POS	1481
1044	POS	2333	POS	2672	POS	5838	POS	1012
1045	POS	3502	POS	2892	POS	7314	POS	1199
1046	POS	2512	POS	3686	POS	7635	POS	2128
1052	NEG	270	POS	6668	NEG	88	POS	3414
1053	NEG	398	POS	7475	NEG	36	POS	3785
1056	POS	2318	POS	3428	POS	7625	POS	2152
1060	NEG	317	POS	7310	NEG	70	POS	3506
1069	POS	3444	NEG	375	POS	8432	POS	2531
1074	POS	3361	NEG	302	POS	8551	POS	2750
1077	POS	3323	NEG	374	POS	8808	POS	2527
1078	POS	2614	NEG	186	POS	7982	POS	1784
1080	POS	1842	POS	3897	POS	7573	POS	2311
1085	POS	2165	POS	3658	POS	8432	POS	2229
1088	POS	3197	NEG	375	POS	9588	POS	2536
1091	POS	1109	POS	3384	POS	7272	POS	1515
1096	POS	2899	NEG	451	POS	8659	POS	1675
1101	POS	2552	POS	4359	NEG	90	POS	2098
1103	POS	3360	POS	2979	POS	9073	POS	1713
1108	POS	2690	POS	4232	POS	6443	POS	2462
1111	POS	3289	NEG	578	POS	8771	POS	1809
1116	POS	3416	NEG	549	POS	9961	POS	1888
1117	POS	3966	NEG	343	POS	9187	POS	2445
1122	POS	3689	NEG	398	POS	10134	POS	2780
1124	POS	2398	POS	4091	POS	8472	POS	2522
1130	POS	2830	POS	4150	POS	7997	POS	2233
1131	POS	3412	NEG	366	POS	8484	POS	2387

1135	POS	4126	NEG	379	POS	9512	POS	2001
1140	POS	3271	POS	3284	POS	8672	POS	2435
1141	POS	4380	NEG	291	POS	9842	POS	2367
1143	POS	3269	POS	3995	POS	8168	POS	3328
1145	POS	3408	POS	3598	POS	8498	POS	2574
1160	POS	3121	POS	4541	POS	9222	POS	2949
1161	NEG	367	POS	6030	NEG	11	POS	3324
1162	POS	2779	NEG	313	POS	8878	POS	1862
1166	POS	3128	POS	3209	POS	8692	POS	2239
1175	POS	3056	POS	2937	POS	6765	POS	2284
1181	POS	3316	NEG	186	POS	9572	POS	3521
1182	POS	2840	NEG	362	POS	8043	POS	3730
1184	POS	2955	POS	3160	POS	6605	POS	2326

2DL5~ 2DS2	2DL5		2DP1		2DS1		2DS2	
	panel	MFI	panel	MFI	panel	MFI	panel	MFI
IHW No. 1003	NEG	459	POS	2429	NEG	66	NEG	432
1010	NEG	152	POS	1178	NEG	21	NEG	93
1016	NEG	157	POS	1255	NEG	21	NEG	85
1017	NEG	162	POS	1317	NEG	56	NEG	97
1018	NEG	189	POS	1531	NEG	15	NEG	104
1021	POS	2016	POS	2364	POS	2792	NEG	323
1029	NEG	428	POS	2091	NEG	49	NEG	345
1031	POS	1735	POS	1710	POS	2693	NEG	298
1040	POS	1636	POS	1417	NEG	61	POS	2847
1044	POS	1306	POS	842	NEG	23	POS	1238
1045	POS	2080	POS	1156	POS	2562	POS	1295
1046	NEG	168	POS	985	NEG	19	POS	1906
1052	NEG	195	NEG	194	NEG	19	POS	5285
1053	NEG	219	NEG	204	NEG	37	POS	5942
1056	NEG	147	POS	821	NEG	29	POS	1871
1060	NEG	139	NEG	183	NEG	30	POS	5230
1069	NEG	170	POS	1781	NEG	25	NEG	95
1074	NEG	197	POS	2085	NEG	45	NEG	100
1077	NEG	176	POS	1359	NEG	27	NEG	81
1078	NEG	155	POS	1790	NEG	31	NEG	84
1080	NEG	171	POS	1266	NEG	22	POS	2082
1085	NEG	136	POS	755	NEG	24	POS	2035
1088	NEG	188	POS	1133	NEG	34	NEG	95
1091	NEG	93	POS	1062	NEG	25	POS	2259
1096	POS	1247	POS	1445	POS	2708	NEG	77
1101	POS	1855	POS	1303	POS	2765	POS	2225
1103	POS	2407	POS	1605	POS	4473	POS	1421
1108	NEG	488	POS	1189	NEG	51	POS	2701

1111	POS	2078	POS	2350	POS	3607	NEG	400
1116	POS	1615	POS	2127	POS	3234	NEG	99
1117	NEG	210	POS	1777	NEG	70	NEG	123
1122	NEG	172	POS	1744	NEG	46	NEG	122
1124	NEG	143	POS	1123	NEG	27	POS	1959
1130	NEG	161	POS	1369	NEG	46	POS	2080
1131	NEG	195	POS	1216	NEG	30	NEG	97
1135	POS	1794	POS	2461	POS	2568	NEG	74
1140	POS	1486	POS	1334	POS	2841	POS	1730
1141	POS	1565	POS	2304	POS	3132	NEG	106
1143	POS	2188	POS	1218	POS	2531	POS	2061
1145	POS	1578	POS	1154	POS	3141	POS	1892
1160	NEG	186	POS	1172	NEG	33	POS	2144
1161	NEG	178	NEG	160	POS	4711	POS	4623
1162	NEG	132	POS	1290	POS	3348	POS	71
1166	POS	2269	POS	1379	POS	2525	POS	1511
1175	POS	1659	POS	893	NEG	33	POS	1501
1181	NEG	286	POS	2066	NEG	41	NEG	228
1182	POS	2677	POS	1683	POS	2448	NEG	209
1184	POS	1985	POS	1378	NEG	51	POS	2174

2DL5~ 2DS2	2DS3		2DS4		2DS5		3DL1	
	panel	MFI	panel	MFI	panel	MFI	panel	MFI
IHW No. 1003	NEG	343	POS	7181	NEG	406	POS	4388
1010	NEG	380	POS	3625	NEG	274	POS	3626
1016	NEG	318	POS	3723	NEG	252	POS	3841
1017	NEG	490	POS	4432	NEG	272	POS	3962
1018	NEG	362	POS	2493	NEG	248	POS	3837
1021	NEG	448	POS	4155	POS	1823	POS	2749
1029	NEG	336	POS	5578	NEG	357	POS	3786
1031	NEG	427	POS	3910	POS	1836	POS	2290
1040	POS	3413	POS	6248	NEG	321	POS	3526
1044	POS	2387	POS	4215	NEG	200	POS	3095
1045	POS	4344	POS	4597	NEG	264	POS	1743
1046	NEG	388	POS	5331	NEG	265	POS	3716
1052	NEG	94	POS	7860	NEG	142	POS	4511
1053	NEG	78	POS	6167	NEG	141	POS	5134
1056	NEG	335	POS	5018	NEG	272	POS	3524
1060	NEG	128	POS	6916	NEG	152	POS	5291
1069	NEG	390	POS	4879	NEG	290	POS	3899
1074	NEG	366	POS	5496	NEG	284	POS	3447
1077	NEG	520	POS	4781	NEG	301	POS	4055
1078	NEG	350	POS	4394	NEG	213	POS	3217
1080	NEG	421	POS	4899	NEG	236	POS	4123

1085	NEG	317	POS	5535	NEG	254	POS	3470
1088	NEG	325	POS	4425	NEG	257	POS	3828
1091	NEG	525	POS	4271	NEG	162	POS	2404
1096	POS	3100	POS	3071	NEG	240	POS	1930
1101	POS	5127	POS	3852	NEG	279	POS	1894
1103	POS	5698	NEG	398	NEG	276	NEG	112
1108	NEG	312	POS	6723	NEG	329	POS	4292
1111	POS	3355	POS	5366	NEG	382	POS	2505
1116	POS	3591	POS	3660	NEG	287	POS	2460
1117	NEG	518	POS	5604	NEG	387	POS	3938
1122	NEG	376	POS	5377	NEG	318	POS	4339
1124	NEG	426	POS	6005	NEG	273	POS	3808
1130	NEG	313	POS	5401	NEG	329	POS	3305
1131	NEG	406	POS	4410	NEG	273	POS	4009
1135	NEG	379	POS	3863	POS	2648	POS	2371
1140	NEG	319	POS	4634	POS	2477	POS	2393
1141	NEG	328	POS	4124	POS	2858	POS	2293
1143	NEG	359	POS	3664	POS	1515	POS	2610
1145	NEG	311	POS	3961	POS	2726	POS	2259
1160	NEG	328	POS	6375	NEG	352	POS	3843
1161	NEG	224	POS	5401	NEG	161	POS	4164
1162	NEG	436	POS	3651	NEG	270	POS	3104
1166	POS	3905	POS	3812	NEG	250	POS	2120
1175	POS	2880	POS	3773	NEG	246	POS	3008
1181	NEG	213	POS	5778	NEG	122	POS	4406
1182	NEG	287	POS	2994	POS	890	POS	2306
1184	POS	3214	POS	6785	NEG	209	POS	3924

3DL2~	3DL2		3DL3		3DP1		3DS1	
3DS1	panel	MFI	panel	MFI	panel	MFI	panel	MFI
IHW No. 1003	POS	9464	POS	8071	POS	8939	NEG	98
1010	POS	7657	POS	7042	POS	6428	NEG	38
1016	POS	9203	POS	7373	POS	6926	NEG	42
1017	POS	9098	POS	7639	POS	7448	NEG	35
1018	POS	8330	POS	7088	POS	7296	NEG	21
1021	POS	7691	POS	7369	POS	8309	POS	1461
1029	POS	7862	POS	7796	POS	8518	NEG	65
1031	POS	8210	POS	7599	POS	7867	POS	994
1040	POS	8582	POS	7904	POS	7560	NEG	87
1044	POS	8613	POS	7129	POS	6556	NEG	27
1045	POS	8212	POS	6882	POS	6577	POS	1225
1046	POS	9184	POS	7997	POS	7461	NEG	45
1052	POS	11173	POS	9825	POS	8404	NEG	81
1053	POS	10186	POS	9793	POS	9166	NEG	51

1056	POS	9124	POS	7807	POS	7443	NEG	15
1060	POS	10953	POS	10399	POS	9126	NEG	53
1069	POS	8861	POS	7836	POS	7045	NEG	56
1074	POS	9165	POS	8138	POS	7363	NEG	33
1077	POS	9384	POS	8141	POS	7659	NEG	25
1078	POS	8704	POS	7672	POS	7599	NEG	37
1080	POS	9062	POS	7652	POS	7638	NEG	31
1085	POS	10162	POS	8067	POS	7653	NEG	23
1088	POS	9575	POS	8488	POS	7706	NEG	29
1091	POS	10289	POS	5865	POS	7457	NEG	16
1096	POS	8475	POS	7020	POS	6704	POS	853
1101	POS	9608	POS	7754	POS	6741	POS	1153
1103	POS	9541	POS	7295	POS	6627	POS	2163
1108	POS	9274	POS	8621	POS	8897	NEG	94
1111	POS	8530	POS	7539	POS	8424	POS	1959
1116	POS	10296	POS	8368	POS	7298	POS	1092
1117	POS	10386	POS	8442	POS	7676	NEG	81
1122	POS	9852	POS	8611	POS	7813	NEG	39
1124	POS	10073	POS	8927	POS	7548	NEG	64
1130	POS	10114	POS	8178	POS	7182	NEG	78
1131	POS	9835	POS	8866	POS	7439	NEG	35
1135	POS	10179	POS	8246	POS	7392	POS	1359
1140	POS	9789	POS	7808	POS	6946	POS	1199
1141	POS	10549	POS	8174	POS	7250	POS	1532
1143	POS	7293	POS	8689	POS	8501	POS	1058
1145	POS	10503	POS	8650	POS	7230	POS	1223
1160	POS	10517	POS	8780	POS	7599	NEG	53
1161	POS	12592	POS	10419	POS	8449	NEG	72
1162	POS	10539	POS	8432	POS	5904	NEG	39
1166	POS	8327	POS	7630	POS	6985	POS	1177
1175	POS	8749	POS	7801	POS	7691	POS	714
1181	POS	9328	POS	8296	POS	8247	NEG	25
1182	POS	4664	POS	7069	POS	7172	POS	861
1184	POS	9046	POS	7757	POS	9577	POS	1281

[+] POS, positive (presence of KIR gene); NEG, negative (absence of KIR gene); MFI, the median fluorescence intensity value of totalplex/bead array-based KIR genotyped sample. MFI values lower than cutoff (600) were marked by gray shadow.

Table 6. Validation of KIR genotyping results of 8 human cell lines by comparing MFI values with SSP-PCR results

2DL1~ 2DL4	2DL1		2DL2		2DL3		2DL4	
	SSP	GSPE	SSP	GSPE	SSP	GSPE	SSP	GSPE
A549	POS	6987	NEG	136	POS	1791	POS	2641
Beas2b	POS	6856	NEG	159	POS	1988	POS	2379
HeLa	POS	3759	POS	4432	NEG	147	POS	2415
H596	POS	7205	NEG	158	POS	1799	POS	2733
MB231	POS	5191	POS	3164	POS	1610	POS	2683
SHSY5Y	POS	7052	NEG	144	POS	2010	POS	2517
U87MG	POS	4995	POS	1655	POS	1528	POS	1946
293	POS	3479	POS	3578	NEG	55	POS	1601

2DL5~ 2DS2	2DL5		2DP1		2DS1		2DS2	
	SSP	GSPE	SSP	GSPE	SSP	GSPE	SSP	GSPE
A549	NEG	231	POS	6964	NEG	103	NEG	118
Beas2b	NEG	226	POS	5385	POS	1663	NEG	106
HeLa	POS	1934	NEG	203	POS	1402	POS	2978
H596	NEG	230	POS	6974	NEG	284	NEG	131
MB231	NEG	185	POS	7123	NEG	166	POS	2557
SHSY5Y	NEG	248	POS	7311	NEG	291	NEG	130
U87MG	POS	2650	POS	6001	POS	1778	POS	1502
293	POS	2298	POS	1404	POS	2890	POS	2539

2DS3~ 3DL1	2DS3		2DS4		2DS5		3DL1	
	SSP	GSPE	SSP	GSPE	SSP	GSPE	SSP	GSPE
A549	NEG	124	POS	1803	NEG	207	POS	3188
Beas2b	NEG	116	POS	1363	NEG	197	POS	2658
HeLa	NEG	123	POS	1403	POS	1420	POS	1970
H596	NEG	108	POS	1771	NEG	212	POS	3414
MB231	NEG	117	POS	1899	NEG	204	POS	3454
SHSY5Y	NEG	113	POS	1827	NEG	205	POS	3442
U87MG	POS	1977	POS	1218	POS	1344	POS	1618
293	POS	5653	POS	4635	NEG	224	POS	1795

3DL2~ 3DS1	3DL2		3DL3		3DP1		3DS1	
	SSP	GSPE	SSP	GSPE	SSP	GSPE	SSP	GSPE
A549	POS	7742	POS	7563	POS	7238	NEG	48
Beas2b	POS	8969	POS	8114	POS	6608	NEG	62
HeLa	POS	8623	POS	7007	POS	6984	POS	2509
H596	POS	8074	POS	7473	POS	7235	NEG	63
MB231	POS	9396	POS	7527	POS	7113	NEG	34
SHSY5Y	POS	8192	POS	7386	POS	7518	NEG	45
U87MG	POS	7573	POS	7031	POS	6424	POS	2485
293	POS	8954	POS	7264	POS	7159	POS	1476

[+] SSP,SSP-PCR; POS, positive (presence of PCR products); NEG, negative (absence of PCR products).

Table 7. Comparison of KIR genotyping results of 8 human buccal samples which were obtained by either SSP-PCR (SSP) or totalplex/bead array-based assay (MFI)

2DL1~ 2DL4	2DL1		2DL2		2DL3		2DL4	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
A	POS	7997	POS	6914	POS	2555	POS	2941
B	POS	9469	POS	3253	POS	1906	POS	2795
C	POS	7074	NEG	101	POS	1594	POS	2593
D	POS	7239	NEG	132	POS	1483	POS	2435
E	POS	8201	POS	5239	POS	2214	POS	2215
F	POS	7211	NEG	205	POS	1774	POS	2538
G	POS	7310	NEG	167	POS	1931	POS	2327
H	POS	7668	POS	8376	POS	2647	POS	2659

2DL5~ 2DS2	2DL5		2DP1		2DS1		2DS2	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
A	NEG	203	POS	6215	POS	3033	NEG	350
B	POS	1491	POS	5520	POS	4349	NEG	180
C	POS	2188	POS	6726	POS	1599	NEG	152
D	POS	1935	POS	6996	POS	1284	NEG	104
E	POS	2119	POS	5037	POS	5670	POS	1710
F	POS	2159	POS	6776	POS	1392	NEG	118
G	NEG	254	POS	7257	NEG	318	NEG	141
H	NEG	190	POS	6647	POS	3555	NEG	152

2DS3~ 3DL1	2DS3		2DS4		2DS5		3DL1	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
A	POS	1257	POS	1443	NEG	292	POS	2467
B	POS	902	POS	1204	POS	2325	POS	1468
C	NEG	134	POS	1433	POS	1132	POS	1892
D	NEG	92	POS	1451	POS	1010	POS	1738
E	POS	5153	POS	1089	NEG	363	POS	1391
F	POS	1850	POS	1577	NEG	197	POS	1940
G	NEG	125	POS	1790	NEG	240	POS	3436
H	POS	1335	POS	1535	NEG	264	POS	2410

3DL2~ 3DS1	3DL2		3DL3		3DP1		3DS1	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
A	POS	11727	POS	8653	POS	6891	NEG	204
B	POS	11189	POS	8448	POS	6511	POS	5598
C	POS	7760	POS	7369	POS	7156	POS	2404
D	POS	7830	POS	7117	POS	6869	POS	2364
E	POS	10771	POS	8298	POS	6336	POS	6061
F	POS	7645	POS	7353	POS	7012	POS	2541
G	POS	8663	POS	7646	POS	7718	NEG	59
H	POS	12859	POS	8594	POS	7076	NEG	414

Table 8. Comparison of KIR genotyping results of 17 peripheral blood samples

2DL1~ 2DL4	2DL1		2DL2		2DL3		2DL4	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
1	POS	3969	NEG	152	POS	9485	POS	3804
2	POS	4604	NEG	204	POS	10348	POS	3132
3	POS	4123	NEG	196	POS	9607	POS	2828
4	POS	4513	NEG	201	POS	9817	POS	2497
5	POS	4617	NEG	200	POS	10272	POS	4042
6	POS	4480	NEG	125	POS	9478	POS	3522
7	POS	4747	NEG	208	POS	9798	POS	3842
8	POS	4718	NEG	204	POS	10727	POS	2466
9	POS	3583	NEG	218	POS	11777	POS	4083
10	POS	4246	NEG	157	POS	9325	POS	2297
11	POS	4456	NEG	281	POS	9743	POS	4743
12	POS	4955	NEG	219	POS	10515	POS	3967
13	POS	5312	NEG	254	POS	11057	POS	3686
14	POS	5106	NEG	292	POS	11446	POS	4623
15	POS	4329	NEG	242	POS	10770	POS	4073
16	POS	3622	POS	3008	POS	8759	POS	3836
17	POS	4216	NEG	205	POS	9800	POS	3464

2DL5~ 2DS2	2DL5		2DP1		2DS1		2DS2	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
1	NEG	359	POS	1003	NEG	27	NEG	173
2	NEG	307	POS	1301	NEG	28	NEG	160
3	NEG	238	POS	1296	NEG	28	NEG	113
4	NEG	238	POS	1286	NEG	35	NEG	114
5	NEG	234	POS	1486	NEG	28	NEG	133
6	NEG	214	POS	1529	NEG	29	NEG	114
7	NEG	270	POS	1482	NEG	35	NEG	108
8	NEG	188	POS	1682	NEG	43	NEG	105
9	NEG	332	POS	733	NEG	36	NEG	174
10	POS	1521	POS	1004	POS	2709	NEG	132
11	NEG	542	POS	1005	NEG	13	NEG	243
12	NEG	349	POS	1881	NEG	47	NEG	177
13	NEG	238	POS	1785	NEG	39	NEG	106
14	NEG	420	POS	1514	NEG	34	NEG	201
15	NEG	358	POS	1344	NEG	28	NEG	149
16	POS	1935	POS	843	NEG	38	POS	1647
17	NEG	349	POS	1388	NEG	25	NEG	156

2DS3~ 3DL1	2DS3		2DS4		2DS5		3DL1	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
1	NEG	168	POS	7825	NEG	161	POS	3953
2	NEG	169	POS	7172	NEG	252	POS	4103
3	NEG	136	POS	7176	NEG	206	POS	4080
4	NEG	254	POS	7770	NEG	215	POS	3585
5	NEG	162	POS	8029	NEG	236	POS	4324
6	NEG	254	POS	7266	NEG	260	POS	3752
7	NEG	200	POS	7387	NEG	247	POS	3976
8	NEG	381	POS	8073	NEG	262	POS	4477
9	NEG	163	POS	6917	NEG	233	POS	4208
10	NEG	401	POS	4781	POS	2079	POS	2065
11	NEG	172	POS	8689	NEG	229	POS	4268
12	NEG	673	POS	7249	NEG	319	POS	4546
13	NEG	353	POS	7649	NEG	351	POS	3908
14	NEG	255	POS	7919	NEG	223	POS	5279
15	NEG	256	POS	8759	NEG	287	POS	4451
16	POS	2669	POS	7761	NEG	195	POS	2404
17	NEG	200	POS	6926	NEG	220	POS	3560

3DL2~ 3DS1	3DL2		3DL3		3DP1		3DS1	
	SSP	MFI	SSP	MFI	SSP	MFI	SSP	MFI
1	POS	7031	POS	1003	POS	8532	NEG	17
2	POS	8151	POS	1301	POS	7402	NEG	20
3	POS	7645	POS	1296	POS	8478	NEG	30
4	POS	8678	POS	1286	POS	7836	NEG	50
5	POS	7425	POS	1486	POS	8788	NEG	26
6	POS	9085	POS	1529	POS	8215	NEG	65
7	POS	7994	POS	1482	POS	7672	NEG	42
8	POS	9616	POS	1682	POS	8281	NEG	41
9	POS	8737	POS	733	POS	9966	NEG	38
10	POS	8158	POS	1004	POS	8334	POS	838
11	POS	6032	POS	1005	POS	8305	NEG	25
12	POS	9345	POS	1881	POS	9310	NEG	68
13	POS	11344	POS	1785	POS	8971	NEG	30
14	POS	9414	POS	1514	POS	9877	NEG	32
15	POS	8750	POS	1344	POS	9190	NEG	35
16	POS	6972	POS	843	POS	8134	POS	1079
17	POS	7609	POS	1388	POS	8877	NEG	20

Table 9. Sensitivity of totalplex/bead array-based KIR genotyping method

samples	amounts (ng/reaction)	2DL1	2DL2	2DL3	2DL4	2DL5	2DP1	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DL3	3DP1	3DS1
HeLa	1	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-
	2	-	+	-	+	+	-	+	+	+	+	-	+	+	+	+	-
	5	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+
	10	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+
	100	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+
293	1	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+	-
	2	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+	-
	5	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
	10	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
	100	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
SH-SY5Y	1	+	-	+	+	-	+	-	-	+	+	-	+	+	+	+	-
	2	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-
	5	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-
	10	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-
	100	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-
peripheral blood	1	+	-	+	+	-	-	-	-	-	+	-	+	+	+	+	-
	2	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-
	5	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-
	10	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-
	100	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-

[+] Data with gray shadow represent false results.

Table 10. KIR gentyping results of pregnant women (M) and their fetus (F)

Samples		2DL1	2DL2	2DL3	2DL4	2DL5	2DP1	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DL3	3DP1	3DS1
Normal	M1	5382	126	7888	3037	170	2440	31	128	319	4691	142	3219	7542	5771	6302	38
	M2	5443	161	7336	2903	161	2642	45	108	475	4783	119	2943	7024	5665	6115	43
	M3	4840	281	8607	2281	2341	2590	4263	103	3688	39	110	93	6496	5274	5312	2621
	M4	5207	103	7869	2933	158	3072	43	101	406	4760	147	2977	7281	5661	5791	83
	M5	5139	89	7862	3228	169	2866	43	92	492	5119	168	2804	7481	5905	5956	53
	M6	5420	117	6892	2356	158	2802	18	106	461	4847	174	2892	7291	5487	5694	34
	M7	3809	2163	7196	1789	1437	1712	3028	1369	2967	3594	113	1987	8084	5982	5081	62
	M8	5557	95	7801	2163	1625	2328	2564	94	557	2856	1555	1811	7547	5631	5691	1312
	M9	5678	190	8736	2388	2325	2102	4477	100	2681	57	1552	108	7149	5381	5630	2485
	M10	6231	129	8145	2592	233	2811	49	130	502	5597	224	3113	7663	5931	6516	64
	M11	5385	125	7240	2540	134	2554	25	91	346	4894	181	2930	7170	5499	5770	34
	M12	4594	233	7562	2296	1770	2465	2645	97	2371	3300	116	1492	6924	5220	5120	1262
	M13	4753	135	7523	3111	171	2771	36	82	344	5219	138	2601	7268	5729	5799	30
	F1	4970	119	7268	2933	184	2517	34	84	361	4715	172	2867	7175	5465	5722	64
	F2	5237	161	7464	3034	157	2354	52	95	435	5295	163	3053	7380	5895	6334	47
	F3	5143	205	7525	1984	1830	2231	2919	102	2826	3729	124	1774	7089	5574	5141	1475
	F4	5737	161	8324	3082	186	2312	57	88	484	4721	176	3165	7592	5874	6440	30
	F5	5836	82	8026	2658	1653	2327	2726	98	399	3420	1420	1644	7334	5372	5848	1337
	F6	4978	134	7840	2429	152	2418	68	86	217	4613	204	2851	7479	5895	5740	56
	F7	5714	112	7055	2665	131	2813	43	79	176	4581	141	2476	6687	5823	5097	62
	F8	6056	129	7969	2345	1926	2688	3027	134	279	3794	1586	1856	7164	5739	5438	1542
	F9	6261	107	8012	2574	2251	2265	4490	104	244	68	2037	93	6735	5492	5406	2683
	F10	5842	137	7916	2929	205	3098	55	108	115	5251	203	3196	8548	6298	6390	50
	F11	5926	142	8391	3332	135	3049	44	88	278	5313	204	3336	8638	6085	6423	35
	F12	5558	258	8169	3070	1926	2697	3280	77	2734	4023	157	1690	7390	5945	6182	1760
	F13	5006	123	7375	3032	1847	2236	3226	80	248	3726	1627	1782	7362	5729	6025	1424
Pre eclampsia	M1	4856	112	6774	2127	1513	1857	2336	151	254	2892	1067	1260	4798	4335	5068	1303
	M2	4721	121	6482	2584	174	2785	38	130	235	4162	180	2470	5625	4857	5496	50
	M3	2946	3094	113	2240	1401	185	3342	2226	156	3585	1168	1498	5759	4903	5108	1990
	M4	4657	127	6874	2680	181	3131	56	132	210	4243	217	2603	5534	5079	5525	52
	M5	5069	142	7989	2717	212	3254	35	128	183	4423	218	2664	6101	5262	5448	45
	M6	4833	146	7296	1922	2255	3053	3625	142	2165	87	1037	127	5071	4456	4915	2677
	M7	5314	100	7457	2474	1768	3389	2628	153	709	3060	1284	1582	5653	5081	5431	1807
	F1	5042	173	7381	3036	270	1761	36	216	261	5019	230	2808	6074	5473	5895	31
	F2	4278	151	7221	3239	254	809	38	207	385	4709	203	2780	5557	5610	5821	44
	F3	4680	3225	7055	2254	1606	1094	55	2238	202	4776	1282	2648	5809	5063	5573	40
	F4	5391	194	7907	3118	273	3181	70	189	359	5029	233	2941	6356	5711	6328	62
	F5	5439	164	8078	3040	270	2166	65	180	229	5280	221	2939	6495	5661	6071	65

[+] KIR genotyping results of F6 and F7 were missing because they were lethal.

Table 11. Distribution of KIR and HLA-C gene haplotypes in nomal (N) and preeclampsia (PE) pregnancy families

	KIR		HLA-C	
samples	mother	fetus	fetus	father
N1	AB	AB	C1/C2	C1/C1
N2	BB	AB	C2/C2	C1/C2
N3	AB	AB	C1/C2	C1/C1
N4	AB	AB	C1/C2	C1/C2
N5	AB	AB	C1/C2	C1/C2
N6	AB	AB	C1/C1	C1/C1
N7	BB	AB	C1/C1	C1/C1
N8	AB	AB	C1/C1	C1/C1
N9	BB	BB	C1/C1	C1/C2
N10	AB	AB	C1/C2	C1/C1
N11	AB	AB	C1/C1	C1/C2
N12	AB	AB	C1/C1	C1/C1
N13	AB	AB	C1/C1	C1/C2
PE1	AB	AA	C1/C1	C1/C1
PE2	AA	AA	C2/C2	C2/C2
PE3	BB	AB	C1/C2	C1/C2
PE4	AB	AA	C1/C2	C1/C2
PE5	AB	AA	C1/C1	C1/C1
PE6	BB	-	-	C1/C1
PE7	AB	-	-	C1/C2

ABBREVIATIONS

NK: Natural Killer

KIR: Killer cell Immunoglobulin-like Receptor

HLA: Human Leukocyte Antigen

MHC: Major Histocompatibility Complex

SBS: Specific Bulge Specific

SSP: Sequence Specific Primer

SSOP: Sequence Specific Oligonucleotide Probes

GSPE: Gene Specific Primer Extension

MFI: Median of Fluorescence Intensity

IHWG: International Histocompatibility Working Group

국문초록

다중유전자 증폭 및 비드 어레이 기술을 이용한 자연살해세포 면역글로불린 유사 수용체 유전형 동정 방법 개발

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박 한 정

자연살해세포 표면에 위치한 면역글로블린 유사 수용체 (Killer cell Immunogloblin-like Receptor, KIR)는 human leukocyte antigen class I 분자와 특이적으로 결합하여 자연살해 세포 활성을 조절하여 건강한 자가 세포를 공격하지 못하도록 하는 역할을 수행한다. KIR 는 총 16 개의 유전자로 구성되어 있고 186 개의 다중유전자 조합을 형성할 수 있는 것으로 알려져 있다. 최근 생식, 감염, 자가면역, 종양과 조혈모세포 이식 등 다양한 영역에서 KIR 유전형과

질병과의 연관성이 보고되면서 KIR 유전형 동정의 중요성이 증가하고 있다. KIR 유전형 동정에는 sequence-specific primer directed polymerase chain reaction (SSP-PCR) 방법이 간단하여 가장 널리 사용되고 있지만 이 방법은 시간과 노동력이 많이 소모되고 μg 단위의 DNA 를 필요로 하며 높은 품질의 DNA 샘플을 필요로 하는 등 여러 가지 단점을 가지고 있다. 따라서 본 연구에서는 좀 더 민감하고 정확하며 분석 시간과 노동력을 줄여줄 수 있는 새로운 KIR 유전형 동정 방법을 개발하고자 하였다. 이를 위해 본 연구에서는 specific bulge specific primer 를 이용한 다중유전자 증폭 (totalplex PCR) 방법을 활용하여 두 번의 PCR 반응을 통해 16 개의 KIR 유전자를 동시에 증폭하였다. 그 후 이 두 가지 증폭 산물을 한 튜브에 섞고 유전자 특이적 프라이머 증폭 기술을 이용하여 2 차 표지 증폭과정을 수행한 후 Luminex 비드 어레이를 이용한 분석을 수행하여 각 DNA 샘플의 KIR 유전형을 분석하였다. 이 방법의 정확성을 검증하기 위해 13th International Histocompatibility Working Group 에서 제공한 48 종의 유전자로 구성된 NK/KIR reference panel I 을 사용한 결과 48 종의 유전형이 정확히 일치함을 확인하였다. 또한 8 종의 세포주, 17 종의 혈액 샘플, 8 종의 협점막 상피 샘플을 이용하여 SSP-PCR 과 본 연구에서 개발한 방법으로 KIR 유전형을 분석한 결과 100% 일치함을 확인하였다. 본 연구에서 개발된 방법의 재현성을 확인하기 위해 동일한 샘플로 6 회 반복 분석한 결과 모두 일치하여 재현성이 높음을 확인하였고, 반응 민감성을

확인하기 위해 다양한 DNA 샘플 양을 사용하여 분석한 결과 5 ng 이상의 DNA 를 사용하면 정확한 유전형 분석이 가능한 것으로 나타났다. 이상의 결과는 새로이 개발된 다중유전자 증폭과 비드 어레이에 기반한 KIR 유전형 동정 방법이 시약, 시간, 노동력, 샘플의 절약 측면에서 매우 유리한 장점을 가지고 있고 재현성이 높으므로 기존의 SSP-PCR 를 대체할 수 있는 우수한 방법임을 시사한다.

주요어: 자연살해세포 면역글로블린 유사 수용체, 유전형 동정, specific bulge specific primer, 다중유전자 증폭, 비드 어레이, 유전자 특이적 프라이머 증폭

학 번: 2005-22456

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감사 드립니다. 지은씨, 혜정씨, 재경씨 부족한 저를 믿고 따라주셔서 감사 드립니다.

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2013년 2월
박 한 정 올림